DESENVOLVIMENTO DE METODOLOGIAS ANALÍTICAS AUTOMÁTICAS EM FLUXO BASEADAS NO CONCEITO DE ESQUEMA REACCIONAL DE INTERFACE ÚNICA

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Cristina Isabel da Cunha Silvestre

RESUMO

O trabalho desenvolvido no âmbito desta tese teve como finalidade explorar a versatilidade e o potencial analítico dos sistemas de fluxo com interface única (SIFA). Desta forma, através da implementação de diversos esquemas reactivos com complexidade variada, envolvendo diferente número de reagentes, diferente tipo de detectores (espectrofotométrico, quimioluminométrico e fluorimétrico) e de sistemas de impulsão (micro-bombas solenóides e bombas de sistemas) e a utilização de fluxos com distintas características hidrodinâmicas (fluxos laminares e pulsados), foi efectuada a avaliação do desempenho e do potencial destes sistemas em distintas situações analíticas.

Neste contexto, utilizando os sistemas SIFA desenvolvidos foi possível implementar o método de Job ou método das variações contínuas para o estabelecimento da estequiometria de complexos, assim como, a avaliação da exactidão baseada na média dos resultados obtidos através de dois métodos *quasi*-independentes originando um resultado mais preciso e exacto. Foi também implementado, pela primeira vez em análise em fluxo, uma extracção aquosa bifásica para a pré-concentração de chumbo. E, num trabalho final, promoveu-se também, pela primeira vez, a determinação quimioluminométrica da carência química de oxigénio baseada em foto-catálise aliada à nanotecnologia de *quantum dots*.

Os sistemas SIFA representam uma nova estratégia de gestão de fluidos, pois não implicam a inserção de volumes definidos de amostra e reagentes nas montagens analíticas, mas sim o estabelecimento de uma interface única de reacção entre a amostra e os reagentes, facto que diverge significativamente do conceito tradicional de análise em fluxo. Desta forma, a concepção, optimização e operação das montagens analíticas é simplificada, em virtude da minimização da influência dos parâmetros volume de amostra e de reagentes no estabelecimento e homogeneização da zona de reacção.

Palavras-chave: Sistema de fluxo com interface única, Quimioluminescência, Espectrofotometria, Fluorimetria, Método de Job, Avaliação da exactidão, Extracção aquosa bifásica, Foto-catálise, *Quantum dots*.

ABSTRACT

The developed work aimed at the exploitation of the versatility and the analytical potential of single interface flow systems (SIFA). Therefore, by implementing several reactive schemes with varying complexity, involving different number of reagents and different kind of detectors (spectrophotometric, chemiluminometric and fluorimetric), and propulsion units (solenoid micro-pumps and piston pumps) as well as flowing streams with distinct hydrodynamic characteristics (laminar and pulsed) the evaluation of the performance of these systems in distinct analytical situations was carried out.

In this context, using the developed SIFA systems it was possible to implement the Job's method or continuous variations method for establishing the stoichiometry of complexes, as well as the accuracy assessment based on the average of the results obtained using two *quasi*-independent methods that lead to a result more precise and accurate. It was also implemented for the first time in flow analysis an aqueous biphasic extraction for preconcentration of lead. And in a final study, it was also evaluated for the first time, the chemiluminometric determination of chemical oxygen demand based on photo-catalysis coupled to *quantum dot* nanotechnology.

SIFA systems represent a new strategy for fluids management, since it does not involve insertion of defined sample and reagents volumes in the analytical manifold, but rather on the establishment of a single interface reaction between the sample and reagents, which differs significantly from the traditional concept of flow analysis. Thus, the conception, optimization and operation of the analytical manifold is greatly simplified simple due to the minimization of the influence of parameters such as volume of sample and reagents in reaction zone implementation and homogenization.

Keywords: Single interface flow analysis, Chemiluminescence, Spectrophotometry, Fluorimetry, Job's Method, Accuracy assessment, Aqueous Biphasic extraction, Photo-catalysis, *Quantum dots*.

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CQO	Carência química de oxigénio
FIA	Análise por injecção em fluxo
MCFS	Sistema de fluxo multi-comutação
MPFS	Sistema de fluxo multi-impulsão
MSFA	Sistema de fluxo multi-seringa
PTFE	Politetrafluoretileno
SIA	Análise por injecção sequencial
SIFA	Sistema de fluxo com interface única
UV	Ultravioleta

CAPÍTULO 1

Introdução Geral

1. Introdução Geral

Os métodos automáticos de análise em fluxo têm apresentado um intenso desenvolvimento e uma acentuada divulgação desde a sua implementação, devido ao baixo custo de instalação, operação e manutenção, elevada versatilidade e facilidade de operação e controlo, reduzido consumo de soluções e elevado ritmo de amostragem proporcionados. A evolução contínua das estratégias de fluxo tem possibilitado a concepção e a introdução de novas modalidades de sistemas e novas estratégias analíticas.

Nas últimas décadas assistiu-se ao desenvolvimento de diferentes métodos de análise em fluxo, como a análise por injecção em fluxo (FIA, do inglês "Flow Injection Analysis") [1], análise por injecção sequencial (SIA, do inglês "Sequential Injection Analysis") [2], sistemas de multi-comutação (MCFS, do inglês "Multicommutation Flow System") [3, 4] e multi-seringa (MSFA, do inglês "Multisyringe Flow Analysis") [5, 6].

Assim, novas metodologias analíticas têm sido desenvolvidas recorrendo a estas diferentes estratégias de gestão de fluidos, na medida em que, permitem a gestão automática de soluções de amostra e reagentes, a implementação de processos reactivos de complexidade variada e o acoplamento de diferentes tipos de detectores. Estruturados de forma diversificada, estes métodos são, no entanto, baseados na utilização de equipamentos com funções semelhantes, nomeadamente um sistema de propulsão (bombas peristálticas, de pistão, de seringa, etc.), um sistema de injecção (válvulas rotativas, solenóides, pneumáticas, multi-selectoras, etc.) e um sistema de detecção (espectrofotómetro, fluorímetro, quimioluminómetro, etc.).

Mais recentemente, foi idealizado e desenvolvido um novo método de análise em fluxo denominado análise em sistemas de fluxo multi-impulsão (MPFS, do inglês "Multi-pumping Flow System") [7]. Os sistemas MPFS geram fluxos com características hidrodinâmicas (fluxos pulsados) distintas das dos métodos de fluxo convencionais (fluxos laminares) e na concepção de montagens analíticas recorrem a um único tipo de elemento activo (micro-bomba solenóide) o qual é responsável, em simultâneo, pela propulsão, inserção e comutação de soluções, obviando o recurso a válvulas de injecção específicas.

Apesar da sua diferente configuração, os fundamentos dos métodos de fluxo são similares: inserção de um volume definido de amostra num fluxo transportador, combinação em linha com soluções de reagentes, e posterior encaminhamento da zona

de reacção, em condições reprodutíveis de dispersão e tempo, para o detector. Esta reprodutibilidade é essencial de modo a que a zona de reacção, em condições de não-equilíbrio químico, produza um sinal transiente proporcional à concentração da espécie em análise.

Independentemente da técnica de fluxo usada, os volumes de amostra e reagentes são parâmetros fundamentais que requerem uma optimização sistematizada, visto que condicionam o estabelecimento da zona de reacção.

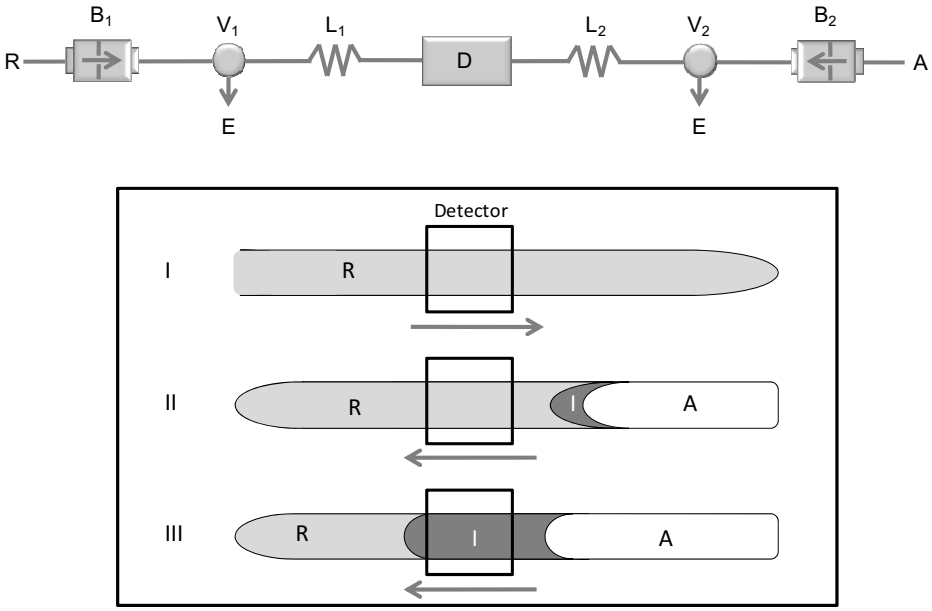
A mais recente estratégia de gestão de fluidos, designada por análise em sistemas de fluxo com interface única (SIFA, do inglês "Single Interface Flow Analysis"), foi proposta por Ribeiro *et al* em 2005 [8], a qual diverge significativamente do conceito tradicional de análise em fluxo. Efectivamente, os sistemas SIFA são caracterizados por não se basearem na inserção de volumes definidos de amostra e reagentes, mas na penetração mútua de zonas de amostra e reagentes numa interface única de reacção, isto é, no estabelecimento de uma interface única de reacção entre a amostra e os reagentes.

A penetração de zonas é a forma mais simples e precisa de se obter uma zona composta. Este conceito foi inicialmente proposto por Ruzicka e Hansen [9] para explicar a formação de uma zona composta quando, em duas zonas adjacentes em movimento contínuo, a primeira sofre uma penetração da segunda, como consequência da velocidade superior do fluxo no centro do tubo, em relação à velocidade média do fluxo.

Nos sistemas SIFA, a dispersão controlada e a formação da zona de reacção, deixam então de ser influenciados pelo volume de amostra e reagentes, passando a ser determinados exclusivamente pela extensão da sobreposição (penetração) de zonas adjacentes de amostra e reagente. Assim, com os sistemas SIFA a sobreposição de zonas nunca é total, uma vez que as zonas de amostra e reagente não têm limites definidos.

Na figura 1 encontram-se esquematizadas duas montagens básicas de um sistema SIFA, uma apresentando o detector numa posição central (A) e outra onde este ocupa uma posição terminal (B). Os dispositivos representados para inserção e propulsão das soluções de amostra e reagente são micro-bombas solenóides. No entanto, convém salientar que os princípios básicos de funcionamento de um sistema SIFA são independentes do sistema de propulsão utilizado e como tal, a implementação destes sistemas não implica a utilização destes dispositivos para a propulsão e inserção de soluções. Para além disso, dependendo das necessidades impostas pela metodologia analítica a implementar, configurações mais complexas podem também ser utilizadas.

(A)



(B)

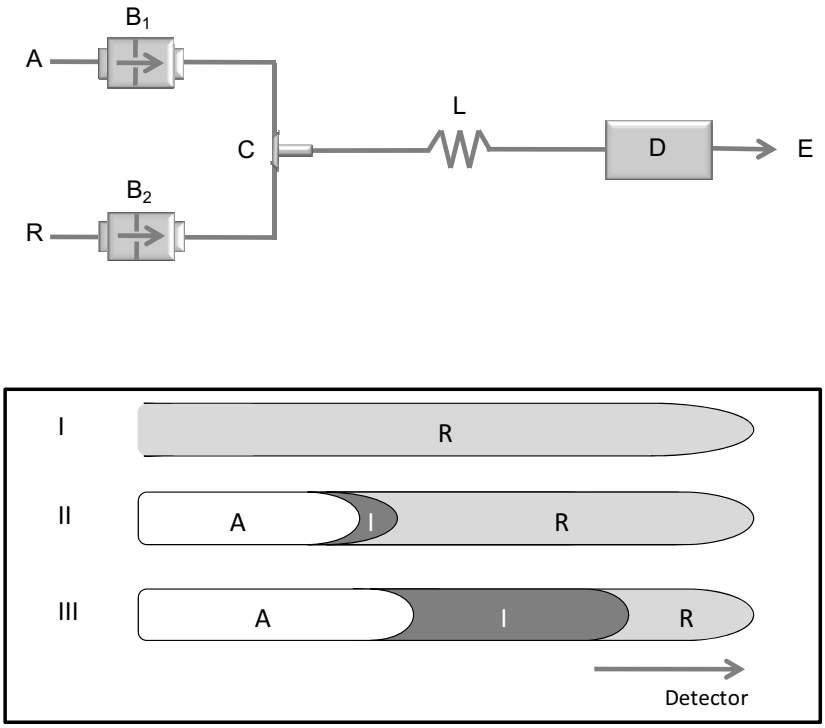


Figura 1 – Representação esquemática de uma montagem SIFA com o detector localizado numa posição central (A) e com o detector localizado numa posição terminal (B) e, respectivo procedimento analítico. B_1, B_2: micro-bombas solenóides; V_1, V_2: válvulas solenóides; L_1, L_2, L: reactor; C: confluência; D: detector; E: esgoto; R: reagente; A: amostra; I: interface única de reacção; I) introdução de reagente; II) inserção de amostra e estabelecimento da interface única de reacção; III) detecção.

O posicionamento do detector no centro da montagem analítica possibilita uma grande variedade de intervenções ao nível da interface de reacção (por acção combinada dos dispositivos de inserção e propulsão das soluções com as válvulas solenóides que direccionam o fluxo), como multi-detecções da zona de amostra através da realização de múltiplas inversões do sentido do fluxo (com ou sem detecção), inversões parciais, renovação de reagentes, monitorização contínua do desenvolvimento de uma reacção, entre outras.

De notar que factores como o recurso a múltiplas inversões do sentido do fluxo e a utilização de um fluxo pulsado poderão ainda contribuir para aumentar o grau de penetração das zonas adjacentes.

No entanto, comparando a montagem analítica que posiciona o detector no centro da montagem com a que apresenta o detector numa posição terminal, verifica-se a existência adicional de duas válvulas solenóides e de um reactor. A presença de um reactor suplementar implica, numa análise, um consumo superior de reagentes e amostra e, em alguns casos, um menor ritmo de determinação, já que é necessário fazer a limpeza de todo o percurso analítico antes de iniciar nova análise.

Visto que os sistemas SIFA são uma estratégia analítica inteiramente nova, o trabalho desenvolvido no âmbito desta tese visou o estudo e caracterização destes sistemas, nomeadamente através da implementação de diversos esquemas reactivos com complexidade variada, envolvendo diferente número de reagentes e diferente tipo de detectores. A avaliação do desempenho e do potencial destes sistemas em distintas situações analíticas foi também explorada.

Assim sendo, o primeiro trabalho desenvolvido (Capítulo 3) envolveu a determinação do manitol baseada, pela primeira vez, na sua actividade "*scavenger*" de radicais hidroxilo. Na montagem analítica do sistema SIFA proposto utilizaram-se micro-bombas solenóides como dispositivos de inserção e propulsão de soluções e um detector quimioluminescente localizado numa posição central. Este sistema foi aplicado à determinação de manitol em formulações farmacêuticas e urina humana.

No Capítulo 4, o sistema SIFA implementado também recorre à colocação do detector, neste caso, espectrofotométrico, no centro da montagem analítica, no entanto, como dispositivos de inserção e propulsão de soluções optou-se por buretas automáticas. Neste trabalho, pela primeira vez, explorou-se a utilização de aceitadores-π para a

determinação das hormonas da tiróide (T_3 e T_4) em formulações farmacêuticas. Para além disso, o sistema desenvolvido permitiu a implementação do método de Job ou método das variações contínuas que teve como finalidade estabelecer a estequiometria dos complexos formados entre os aceitadores-π e respectiva hormona da tiróide.

Os Capítulos 5 e 6 apresentam trabalhos cujas montagens desenvolvidas colocam o detector numa posição terminal, bem como, utilizam micro-bombas solenóides como dispositivos de inserção e propulsão de soluções.

Através do trabalho apresentado no Capítulo 5, o conceito de SIFA foi expandido ao estabelecer duas interfaces de reacção que foram aplicadas na determinação espectrofotométrica de lansoprazol após reacção com dois aceitadores-π. A média dos resultados obtidos por estes dois métodos espectrofotométricos *quasi*-independentes permitiu um resultado mais preciso e exacto, mostrando a viabilidade de um sistema SIFA com avaliação da exactidão ("*accuracy assessment*"). A formação de complexos entre o lansoprazol e os aceitadores-π foi explorada pela primeira vez para a determinação espectrofotométrica deste analito em formulações farmacêuticas.

Demonstrando mais uma vez a fiabilidade, a versatilidade e o potencial analítico dos sistemas SIFA, o Capítulo 6 apresenta um trabalho no qual foi implementado, pela primeira vez em análise em fluxo, uma extracção aquosa bifásica. Este tipo de extracção é extremamente atractivo em termos ambientais, pois é baseada na utilização de duas fases aquosas imiscíveis, não fazendo uso dos solventes orgânicos tão comuns nas extracções líquido-líquido convencionais. O sistema SIFA desenvolvido permitiu a pré-concentração de chumbo e, após a extracção, este analito reagia com ácido 8-hidroxiquinolina-5-sulfónico, cujo produto de reacção era determinado através de um detector fluorimétrico incluído na montagem analítica. Assim, a interface única estabelecida foi utilizada quer como interface de extracção, quer como interface de reacção.

Finalmente, o capítulo 7 reporta um novo método para a determinação da carência química de oxigénio (CQO). Neste trabalho, pela primeira vez, a capacidade dos *quantum dots* de CdTe para gerar espécies oxidantes, quando irradiados com luz UV, é utilizada para promover a foto-catálise de várias moléculas orgânicas. Assim, o sistema SIFA implementado incluía na montagem analítica uma unidade de foto-irradiação e um detector de quimioluminescência, localizado numa posição central, para monitorizar a luz emitida após a oxidação do luminol pelas espécies oxidantes geradas durante a irradiação dos *quantum dots*. Assim, o CQO é avaliado indirectamente. O sistema SIFA

proposto foi aplicado à determinação de CQO em material de referência certificado. Esta abordagem permite obviar vários problemas associados com a metodologia oficial para a determinação de CQO, nomeadamente, longo tempo de análise, operações fastidiosas e consumo de reagentes dispendiosos e tóxicos.

Os sistemas SIFA encontram-se ainda numa fase inicial de avaliação e desenvolvimento, mas as suas características operacionais e os resultados já obtidos permitem antever um elevado potencial de aplicação desta metodologia, a qual poderá constituir uma alternativa vantajosa não só aos métodos discretos utilizados mas também relativamente às técnicas de fluxo convencionais.

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CAPÍTULO 2

Aspectos Gerais da Parte Experimental

2. Aspectos gerais da parte experimental

2.1. Introdução

Neste capítulo são descritos os aspectos experimentais comuns aos trabalhos desenvolvidos. São assim referidos os procedimentos gerais utilizados na preparação das soluções, assim como, os equipamento utilizados na concepção das montagens de fluxo, salientando-se as suas características e modo de operação e controlo. São ainda apresentados os procedimentos de optimização seguidos no desenvolvimento das metodologias propostas e o tratamento estatístico utilizado na avaliação da qualidade dos resultados obtidos.

2.2. Reagentes e soluções

Na preparação das soluções usaram-se reagentes de qualidade *p.a*. ou semelhante, os quais não foram sujeitos a qualquer tratamento ou purificação adicional.

A água desionizada utilizada na preparação de soluções, com condutividade inferior a 0,1 μS cm^{-1}, foi obtida por passagem em resinas de troca iónica de leito misto, incorporadas num aparelho de desionização da marca Milli-Q, modelo RG.

As soluções padrão concentradas foram obtidas por pesagem rigorosa do respectivo reagente numa balança analítica (modelo AG 285, Mettler Toledo), seguida de dissolução do mesmo em solvente adequado, em material de vidro de classe A. As soluções padrão envolvidas no traçado das curvas de calibração foram obtidas por diluição rigorosa da solução padrão concentrada, utilizando pipetas volumétricas de diferentes volumes ou pipetas automáticas (modelo LM100, LM1000 e LM5000, LabMate HTL) e balões volumétricos.

2.3. Componentes dos sistemas de fluxo

Os sistemas de fluxo implementados foram concebidos com recurso a diferentes tipos de equipamento, com diferentes características operacionais e com funções específicas

na montagem analítica, nomeadamente para inserção, propulsão, comutação e transporte das soluções, detecção e registo do sinal analítico.

2.3.1. Dispositivos de propulsão e inserção

Nos sistemas de fluxo desenvolvidos foram utilizados dois tipos de dispositivos de propulsão e inserção de soluções: micro-bombas solenóides e buretas automáticas.

Nas montagens analíticas envolvendo a utilização de micro-bombas solenóides foram usadas micro-bombas da marca BioChem Valve Inc. (Boonton, EUA) com um volume de pulso fixo de 10 μL (Capítulos 3, 5 e 7) e 20 μL (Capítulo 6), modelo 120SP.

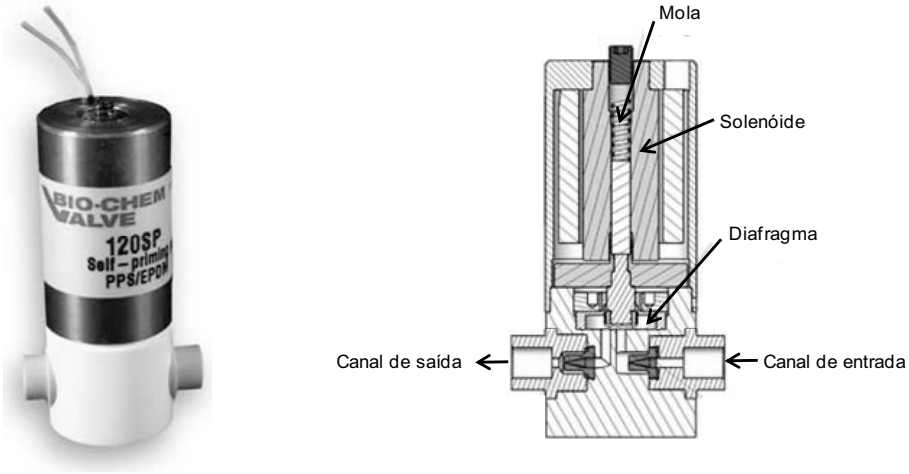

Figura 2 – Micro-bomba solenóide e respectiva representação esquemática do seu interior.

O funcionamento das micro-bombas é baseado no deslocamento de um diafragma por activação de um solenóide (figura 2). Na posição de repouso, o diafragma é mantido na posição fechada por intermédio de uma mola interna. Por aplicação de uma voltagem o solenóide é activado o que provoca o deslocamento do diafragma e a aspiração do líquido para uma câmara interna. Quando a voltagem é suprimida o solenóide é desactivado e a mola interna recoloca o diafragma na sua posição inicial. A acção da mola sobre o diafragma, num movimento súbito, provoca a propulsão do líquido contido na câmara, gerando um pulso de volume fixo correspondente ao seu volume interno.

Para um funcionamento ideal, as micro-bombas requerem a aplicação de uma voltagem de 12 V, durante um período de pelo menos 150 ms (aspiração das soluções), o qual é seguido de um período mínimo de desactivação do solenóide de cerca de 350 ms (propulsão das soluções). A soma do período de activação e de desactivação do solenóide define o tempo de pulso. Apesar dos valores indicados, as micro-bombas permitem a utilização de tempos de activação e de desactivação na ordem dos 100 ms, o que permite atingir frequências de pulso de 300 pulsos por minuto. Para cada solução, a frequência de pulso em combinação com o volume de pulso da micro-bomba determina o caudal, e o número de pulsos define o volume inserido no percurso analítico.

Na montagem analítica envolvendo a utilização de buretas automáticas (Capítulo 4) foram utilizadas duas buretas da marca Crison (Alella, Espanha), modelo micro BU 2031, equipadas com seringas de vidro da marca Hamilton com a capacidade de 5,0 mL (modelo 1005).

O funcionamento das buretas é baseado no movimento de um êmbolo operado por um motor de passo. O curso completo do êmbolo da seringa corresponde a 2500 passos, e cada passo a um volume de 2 μL. Para cada solução, o tempo de passo determina o caudal e, o número de passos define o volume introduzido na montagem analítica.

2.3.2. Dispositivos comutadores

Nas montagens estabelecidas, com excepção da descrita no Capítulo 5, foram utilizadas válvulas solenóides de três vias (duas entradas/uma saída), da marca NResearch Inc. (West Caldwell, USA), modelo 161 T031, como dispositivo comutador, com a finalidade de direccionar o fluxo.

Nestas válvulas, a selecção do percurso das soluções é condicionada pela posição de duas membranas de politetrafluoretileno (PTFE) presentes na parte central da válvula (figura 3). Assim, um dos percursos é seleccionado através da activação do solenóide, tendo como consequência a aplicação de uma pressão numa das membranas, ao passo que o percurso alternativo é estabelecido após o desligar da válvula, através do deslocamento de uma mola helicoidal que permite a reposição da membrana na sua posição inicial. Deste modo, a válvula solenóide funciona como um interruptor, apresentando dois estados possíveis, permitindo que, em momentos diferentes, a válvula seja activada e desactivada, alternadamente.

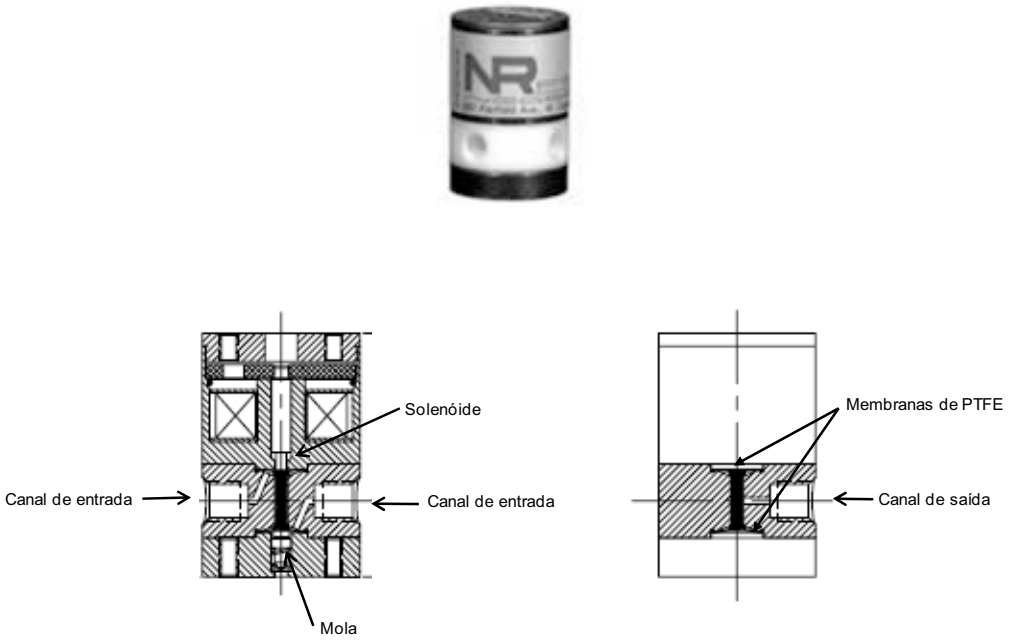

Figura 3 – Válvula solenóide de três vias e respectiva representação esquemática do seu interior.

Estas válvulas apresentam reduzidos volumes mortos; reduzido volume interno; pressão máxima de funcionamento de 30 psi; curto tempo de resposta (5 a 20 ms para activação); elevada resistência química (revestimento em PTFE) e alimentação a 12 V.

2.3.3. Tubagem e outros componentes da montagem

O sistema de tubagem para o transporte das soluções era constituído por tubos de PTFE, da marca Omnifit, com um diâmetro interno de 0,8 mm, ligados entre si por ligadores de fabrico próprio. Os reactores, com diferentes configurações (linear, espiral e figura “8”) foram construídos com tubos do mesmo diâmetro e da mesma marca. As confluências utilizadas eram de construção laboratorial e fabricadas em perspex.

2.3.4. Sistemas de detecção

Nos capítulos 3 e 7 utilizou-se um luminómetro da marca Camspec modelo CL-2, equipado com uma célula de fluxo com um volume interno de 60 μL. A célula de fluxo possuía duas portas de entrada e uma de saída, podendo as duas portas de entrada ser utilizadas para a inserção das soluções de amostra e reagente, o que possibilitava a mistura das soluções mesmo à entrada da célula de fluxo, podendo também ser utilizada apenas uma das entradas, fechando a outra e efectuando-se a mistura antes da célula de fluxo. Este último caso foi o utilizado nas metodologias desenvolvidas.

No capítulo 4, o sistema de detecção utilizado foi um espectrofotómetro da marca Jenway, modelo 6305, equipado com uma célula de fluxo, da marca Hellma, com um volume interno de 80 μL e 1 cm de percurso óptico.

A montagem analítica desenvolvida no capítulo 5 incluía um espectrofotómetro da marca OceanOptics (Dunedin, USA) modelo USB 2000, equipado com uma célula de fluxo acrílica em forma de Z com um volume interno de 10 μL e 1 cm de percurso óptico.

No capítulo 6, o sistema de detecção utilizado foi um espectrofluorímetro da marca PerkinElmer, modelo LS-30, equipado com uma célula de fluxo com um volume interno de 7 μL.

Os sinais analíticos eram registados por intermédio de um registador da marca Kipp & Zonen, modelo BD111, que se encontrava conectado ao sistema de detecção, ou adquiridos directamente para o computador através de uma interface com um conversor analógico/digital com uma resolução de 12 bits.

2.3.5. Controlo informático dos sistemas

Os sistemas analíticos desenvolvidos eram controlados integralmente por computador, através de software desenvolvido no laboratório, possibilitando a selecção e ajuste, de forma simplificada, de todos os parâmetros analíticos que condicionavam o desempenho das montagens.

Como unidade central de aquisição e processamento de dados e de controlo dos componentes dos sistemas desenvolvidos foi utilizado um microcomputador equipado com um microprocessador Pentium I. A comunicação entre o microcomputador e os

diversos componentes das montagens de fluxo, nomeadamente os dispositivos de propulsão e inserção (micro-bombas solenóides e buretas automáticas), dispositivos comutadores (válvulas solenóides) e dispositivos de detecção (luminómetro, espectrofotómetros e espectrofluorímetro) foi efectuada através de uma interface da marca Advantech, modelo PC-LabCard PCL-71 1 B.

Para a actuação das micro-bombas solenóides e das válvulas solenóides foi desenvolvido um circuito de potência, com base num circuito integrado ULN 2003 [1]. Este circuito permitia o controlo simultâneo de sete unidades (micro-bombas solenóides e válvulas solenóides) e era activado por sinais TTL ("*Transistor-Transistor Logic*") através da interface anteriormente referida.

As buretas automáticas eram controladas por comunicação série usando o padrão RS-232C.

O software de controlo, aquisição e tratamento de dados, foi elaborado especificamente para aplicação nos trabalhos realizados. As aplicações foram desenvolvidas com recurso a uma linguagem de alto nível, BASIC, implementada em ambiente DOS (QuickBasic 4.5).

De um modo geral, cada programa permitia definir a sequência de etapas para cada determinação. Em cada caso particular as instruções dadas referiam-se à activação das micro-bombas solenóides (tempo de pulso, frequência de pulso e número de pulsos) e à activação das buretas automáticas (sentido de funcionamento aspiração/impulsão, tempo de activação e número de passos) e ainda à posição das válvulas solenóides.

2.4. Instrumentação adicional

As medições espectrofotométricas realizadas nas experiências preliminares referentes aos trabalhos desenvolvidos nos capítulos 3 e 4 foram realizadas utilizando um espectrofotómetro de UV/Vis, da marca PerkinElmer, modelo Lambda 45.

A determinação de lansoprazol em formulações farmacêuticas (Capítulo 5) foi realizada seguindo o método de referência descrito na Farmacopeia Americana [2], utilizando um cromatógrafo da marca Jasco, modelo LC-Net II ADC, equipado com um detector MD-2015 Plus e uma bomba PU-2080 Plus.

A montagem analítica do sistema SIFA desenvolvido para a determinação de CQO (Capítulo 7) incluía uma lâmpada UV de 15 W com λ_{max} de 256 nm.

Sempre que era necessário ajustar o pH das soluções recorria-se um milivoltímetro da marca Crison, modelo GLP 22 equipado com um eléctrodo de vidro combinado de Ag/AgCI, da mesma marca e com a referência 52-02.

2.5. Desenvolvimento e optimização das montagens de fluxo

No desenvolvimento dos sistemas SIFA apresentados foram realizados vários estudos com o objectivo de melhorar o seu desempenho, nomeadamente em termos de sensibilidade, ritmo de amostragem, precisão, exactidão e consumo de amostras e reagentes. Estas características influenciaram as escolhas efectuadas durante a optimização destes sistemas.

Para além disso, estes estudos de optimização foram realizados recorrendo ao método da análise univariada, o qual consistia em fazer variar o valor de um determinado parâmetro mantendo os restantes com um valor constante.

O intervalo de concentrações em que se verificava uma relação linear entre o sinal analítico e a concentração do analito foi definido através do traçado de curvas de calibração, com soluções de concentração crescente do analito a determinar, e pelo estabelecimento do intervalo de concentração no qual se observava essa relação linear.

O ritmo de amostragem ou o ritmo de determinação, expresso em número de amostras ou determinações por hora, respectivamente, foram calculados tendo em consideração o tempo necessário para a realização de todas as etapas do ciclo analítico.

2.6. Tratamento estatístico dos resultados

O sinal analítico, correspondente à concentração da espécie a analisar, foi calculado em função da média de 3 medições consecutivas da mesma amostra. A concentração final era depois calculada por interpolação gráfica da intensidade ou amplitude do sinal obtido, na curva de calibração estabelecida para cada metodologia.

A avaliação da precisão das metodologias implementadas foi efectuada por ensaio das soluções padrão e das amostras. Para tal foram realizadas 3 determinações consecutivas e foi determinado o desvio padrão relativo.

A exactidão das metodologias desenvolvidas foi avaliada por comparação dos resultados obtidos com o sistema SIFA implementado com os determinados pelo método de referência, sempre que possível (Capítulos 3 e 5). Para cada determinação calcularam-se os desvios relativos, expressos em percentagem, para o valor médio de cada par de resultados obtidos, com base na expressão $(C_{SIFA} - C_{Ref}) \times 100 / C_{Ref}$, onde C_{SIFA} é a concentração obtida pela metodologia SIFA e C_{Ref} é a concentração obtida através da metodologia de referência. Foi também aplicado o teste *t* de Student emparelhado (*paired t-test*) aos valores obtidos pelas duas metodologias. O valor calculado ($t_{calculado}$) quando comparado com o valor tabelado ($t_{tabelado}$), para um nível de significância de 95%, permite indicar a ausência de diferenças estatisticamente significativas entre as médias das concentrações obtidas pelos dois métodos, confirmando a concordância entre os dois métodos quando se verifica a hipótese nula [3].

Outra forma de avaliar a exactidão dos resultados obtidos pelas metodologias desenvolvidas foi através de ensaios de recuperação (Capítulos 3 e 4). Estes ensaios foram efectuados adicionando a cada amostra, soluções padrão concentradas da espécie em análise, de modo a obter dois níveis finais de concentração. A percentagem de recuperação foi calculada com base na expressão $(C_{a+p} - C_a) \times 100 / C_p$, onde C_{a+p} é a concentração do analito obtida na amostra em que foi adicionada solução padrão, C_a é a concentração do analito obtida na amostra e C_p é a concentração do analito na amostra, resultante da adição da solução padrão.

Na metodologia desenvolvida no Capítulo 7, a exactidão foi avaliada através da análise de amostras de referência certificadas.

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CAPÍTULO 3

Desenvolvimento de um sistema de fluxo com interface única para a monitorização quimioluminescente do manitol baseada na sua actividade "*scavenger*" de radicais hidroxilo

Neste trabalho foi desenvolvido um sistema SIFA para realizar a monitorização do manitol em formulações farmacêuticas e em urina humana, sem qualquer tipo de pré-tratamento das amostras. O manitol é vastamente usado na indústria alimentar e farmacêutica, assim como, apresenta um importante papel terapêutico actuando como diurético osmótico.

A abordagem proposta para a determinação do manitol envolveu a utilização de um sistema mioglobina-luminol que originava a formação em linha de espécies químicas com um tempo de vida muito curto – radicais hidroxilo. A mioglobina no seu estado férrico (Fe^{3+}) oxidava o luminol em meio alcalino promovendo quimioluminescência. A reacção do Fe^{3+} da mioglobina com o NaOH gerava radicais hidroxilo e Fe^{2+}. Assim, a emissão quimioluminescente era consequência da oxidação do luminol induzida pelos radicais livres originados.

Uma vez que o manitol é um "*scavenger*" específico de radicais hidroxilo, através desta capacidade, a inibição da reacção mencionada entre a mioglobina e o luminol era utilizada para a determinação do manitol.

O sistema SIFA implementado permitiu, desta forma, a determinação do manitol com concentrações compreendidas entre 25 mmol L^{-1} e 1 mol L^{-1} e uma frequência de amostragem de 60 h^{-1}. Os resultados obtidos para as formulações farmacêuticas foram estatisticamente comparáveis com os alcançados pelo método de referência (titulação iodométrica), facto verificado pela aplicação do teste *t* de Student emparelho, e os valores dos ensaios de recuperação obtidos na análise de urina humana foram também satisfatórios.

Convém ainda salientar que é relevante não só o facto de ter sido a primeira vez que se utilizou a capacidade "*scavenger*" do manitol para a sua determinação, mas também a utilização de um sistema SIFA que permitiu a rápida, simples e fiável implementação desta determinação.

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Single reaction interface flow system for chemiluminescent monitoring of mannitol based on its hydroxyl radical scavenger activity

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ABSTRACT

A single reaction interface flow analysis (SIFA) system for the monitoring of mannitol in pharmaceutical formulations and human urine is presented. The developed approach takes advantage of the mannitol scavenger aptitude to inhibit the chemiluminescent reaction between luminol and myoglobin in the absence of H_2O_2. The SIFA system facilitated the fully automation of the developed methodology, allowing the in-line reproducible handling of chemical species with a very short lifetime as is the case of the hydroxyl radical generated in the abovementioned luminol/myoglobin reaction.

The proposed methodology allowed the determination of mannitol concentrations between 25 mmol L^{-1} and 1 mol L^{-1}, with good precision (R.S.D. < 4.7%, $n = 3$) and a sampling frequency of about 60 h^{-1}. The procedure was applied to the determination of mannitol in pharmaceuticals and in human urine samples without any pretreatment process. The results obtained for pharmaceutical formulations were statistically comparable to those provided by the reference method (R.D. < 4.6%); recoveries values obtained in the analysis of spiked urine samples (between 94.9 and 105.3% of the added amount) were also satisfactory.

1. Introduction

Myoglobin is a small and stable heme protein which contains a single iron protoporphyrin or heme moiety and thus classified as a metalloprotein [1]. It is mainly present in both skeletal and cardiac muscle tissue where it is responsible for oxygen storage and transportation. Several recent works refer that myoglobin in the ferric state Mb(Fe^{III}) could oxidise luminol yielding a chemiluminescence emission. This feature was applied in the determination of several species that could either inhibit or enhance the myoglobin–luminol system [2–4].

It has been also reported that some metal-containing compounds and metalloporphyrins could produce a chemiluminescence response from luminol in alkaline medium, without involving the direct oxidation of the later by myoglobin or the presence of hydrogen peroxide [5]. Seemingly, the reaction between the Fe^{III} porphyrins with NaOH generated hydroxyl radical ($OH^{\bullet}$) and Fe^{II} porphyrins, which could be subsequently oxidised to the Fe^{III} form by O_2 generating superoxide radical ($O_2^{\bullet -}$) [5]. The chemiluminescence emission was therefore a consequence of luminol oxidation induced by the free radicals generated in the redox cycle. Being a metalloporphyrin it would be expected that myoglobin would endure a similar chemical process under the same conditions.

Mannitol is a polyol (sugar alcohol) widely used in food and pharmaceutical industries as well as a therapeutical agent (osmotically active diuretic) used in situations of acute renal failure, to treat cerebral oedema, glaucoma or to reduce intracranial pressure [6]. Mannitol has been determined in different matrices including food, biological samples or pharmaceuticals mainly by chromatography [7–20] and capillary electrophoresis [21–26], using various types of detection, such as, UV, spectrophotometric, electrochemical, amperometric, conductimetric and refractometric. However, the majority of these techniques is time-consuming and requires expensive equipments and qualified operators. Two flow injection analysis (FIA) methodologies involving chemiluminescence detection upon oxidation by permanganate [27] and fluorometric detection [28], were also proposed for the determination of this polyol. However, FIA systems exhibit a low automation level and rely on the continuous addition of reagent solutions to the sample zone, which lead to a substantial consumption of reagents. Furthermore, FIA versatility is limited in terms of sample manipulation since the inserted sample volume is determined by the internal volume of the sample loop.

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Given that mannitol is a specific and strong hydroxyl radical scavenger it would have the ability, by scavenging ($OH^{\bullet}$), to inhibit the anticipated chemiluminescence response generated in the abovementioned myoglobin/luminol reaction, a capability that could be used for its determination in samples with distinct origins. It is relevant the fact that none of the methodologies proposed till now makes use of mannitol radical scavenger capability for its determination.

Chemiluminescence measurements are characterised by wide dynamic linear ranges, high speed of response and excellent sensitivity. Taking into account that a CL response is typically generated by fast reactions, its efficient monitoring requires highly reproducible and fast mixing of sample and reagents. This is even more crucial when short-lived species, as is the case of the hydroxyl radical, are involved in the reactive process. SIFA systems exhibit all the necessary features to fulfil these requisites. Therefore, in this work an automated flow methodology based on a single reaction interface flow analysis (SIFA) system [29] was implemented for monitoring mannitol levels in pharmaceutical and biological samples. The use of solenoid micro-pumps which are accountable for solutions insertion, propelling and commutation, conditioning the establishment and subsequent detection of the reaction zone, provide a great operational simplicity. These micro-pumps are ideal tools to build up compact environmentally friendly analytical systems, which are characterised by low solutions consumptions and the minimisation of hazardous waste generation. They also enhanced sample/reagent mixing ability in comparison with flow techniques that rely on laminar flow regime. Effectively, solenoid micro-pumps actuation produces a pulsed flowing stream as a consequence of the sudden pump diaphragm displacement that produces a chaotic movement of the solutions in all directions leading to the improved sample/reagent mixing, a well-known requirement of chemiluminescence measurements (fast and effective sample/reagent mixing). Nonetheless, one of the main advantages of SIFA systems is that they no longer rely on the utilisation of well-defined and compelling sample and reagent volumes, a typical characteristic of conventional flow techniques such as FIA, Sequential Injection Analysis (SIA) and Multi-Syringe Flow injection Analysis (MSFIA), but on the establishment of a single sample/reagent reaction interface, where the sample and reagent solutions have no fixed boundaries. This facilitates system configuration and control and resulted in enhanced simplicity and operational versatility while minimises the occurrence of operational errors.

2. Materials and methods

2.1. Reagents and solutions

All of the solutions were prepared with water from a Milli-Q system (conductivity $\leq 0.1\ \mu S\,cm^{-1}$) and chemicals were of analytical reagent grade quality and not subject to any purification.

A $1.0 \times 10^{-3}\ mol\,L^{-1}$ luminol solution was daily prepared dissolving 8.86 mg in 50 mL of NaOH 0.2 mol L^{-1}. Working standard solutions were prepared by suitable dilutions with NaOH 0.2 mol L^{-1}.

A 1.0×10^{-5} mol L^{-1} myoglobin solution was daily prepared dissolving 8.48 mg in 50 mL of water. Working standard solutions were prepared by suitable dilutions with water.

Working standard solutions of mannitol were daily prepared by rigorous dilution of 1 mol L^{-1} stock solution using water.

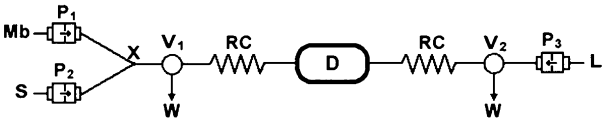

Fig. 1. Single interface flow manifold for the determination of mannitol. P_1, P_2, P_3: solenoid micro-pumps; X: confluence point; V_1, V_2: solenoid valves; RC: reaction coils (50 cm); D: chemiluminescence detector; W: waste; Mb: myoglobin (4×10^{-7} mol L^{-1}); S: sample or standard; L: luminol (1×10^{-5} mol L^{-1}; prepared in NaOH 0.2 mol L^{-1}).

2.2. Apparatus

The single interface flow system comprised three solenoid micro-pumps (120SP1210-4TE, Bio-Chem Valve Inc., Boonton, NJ, USA, 10 μL per stroke), two 161 T 031 (NResearch, West Caldwell, USA) two-way solenoid valves and a Camspec CL-2 chemiluminescence detector (Camspec Ltd., Cambridge, UK) equipped with a three-port 60 μL inner volume quartz flow cell. The luminometer had a wavelength response range of 320–600 nm and a flow cell working pathlenght of 5 mm. Flow lines and reaction coils were made from 0.8 mm i.d. PTFE tubing.

A Pentium-I-based computer was used for system control, and for data acquisition and treatment; software was developed in Microsoft Quick-Basic 4.5. The computer was equipped with a PC-LABCard model PCL-711B interface card from Advantech (Taipei, Taiwan). A CoolDrive (NResearch Inc., West Caldwell, USA) power drive was used to operate both the solenoid micro-pumps and solenoid valves.

Spectrophotometric measurements were carried out in a UV/Vis Spectrometer model Lambda 45 from PerkinElmer Instruments Inc. (Norwalk, CT, USA).

2.3. Single interface flow manifold

The developed flow manifold, pictured in Fig. 1, comprised three solenoid micro-pumps (P_1, P_2 and P_3), which were used to insert and propel the sample and reagent solutions. The repetitive micro-pump switching on/off created a pulsed flowing stream in which the pulse volume corresponded to the micro-pump stroke volume. Two two-way (normally closed) solenoid valves (V_1 and V_2) were used to direct the flowing streams. The detector was placed at the centre of the flow manifold. The reactions coils, identically sized, were placed on both sides of the detector.

The analytical cycle was started by establishing a baseline, which was accomplished with myoglobin/sample solutions. For establishing the baseline, V_1 was open and P_1 and P_2 were repeatedly actuated (on/off switching) propelling the solutions through the reactors as well as through the detector. After reaching V_2 these solutions were discarded. Subsequently, V_2 was opened, P_1 and P_2 were switched off and the luminol solution was inserted into the analytical path by means of P_3 and was propelled back to V_1. The mutual interdispersion of myoglobin/sample/luminol resulted in an analytical signal, which was measured when the reaction interface passed through the detector.

2.4. Reference method

Aiming at the evaluation of the accuracy of the results obtained with the developed procedure, mannitol pharmaceutical formulations were analysed according to the British Pharmacopoeia [30], by iodimetric titration.

3. Results and discussion

Preliminary experiments showed that at pH < 8 myoglobin exhibits a maximum of absorbance at 409 nm, corresponding to $Mb(Fe^{III})$ form (metmyoglobin), while at higher pH values the peak maximum was at 414 nm for $Mb(Fe^{II})$ (oxymyoglobin). It was also observed that the ferric state myoglobin reacted with luminol, in alkaline medium, producing a strong chemiluminescence (CL) emission. This CL emission was markedly reduced when mannitol, a strong and specific hydroxyl radical scavenger, was added confirming that this free radical was involved in the production of light. These results agreed with previous results obtained with other iron porphyrins [5].

3.1. Development of single interface flow methodology

In the development of the single interface flow methodology several studies were carried out in order to improve systems performance, namely in terms of analytical signal intensity, accuracy, repeatability and determination rate. These features influenced the choices made during the optimisation of the systems, which was carried out using the univariate method. Since no well-defined sample or reagent volumes were used, these parameters were not subject to evaluation which simplified the overall optimisation process.

3.1.1. Chemical parameters

Considering that the pH of the solution determined the Fe oxidation state, in order to guarantee the $Mb(Fe^{III})$ form myoglobin solutions were prepared directly in deionised water at pH 5.6. Effectively, when myoglobin was prepared in alkaline medium a weak CL intensity was observed. On the other hand, luminol CL emission occurred in alkaline conditions. Under these circumstances the SIFA system would have to provide good mixing conditions at the myoglobin/luminol single interface to ensure an appropriate reaction development.

The influence of myoglobin concentration in the chemiluminescence intensity was assayed for concentrations ranging from 1.0×10^{-7} to 6.0×10^{-7} mol L^{-1}. It was observed that with increasing myoglobin concentrations the intensity of the signal increased about 61.5% until myoglobin 4.0×10^{-7} mol L^{-1}, above which the chemiluminescent signal approached stabilisation. Therefore, a 4.0×10^{-7} mol L^{-1} myoglobin solution was used in the subsequent experiments.

The chemiluminescence intensity was also studied for luminol concentrations between 0.1×10^{-5} and 5×10^{-5} mol L^{-1}. The obtained results showed that with increasing luminol concentrations the intensity of the chemiluminescent response increased about 59.1% until 1.0×10^{-5} mol L^{-1}. Above this concentration value the chemiluminescent intensity remained almost unaffected. Consequently, a 1.0×10^{-5} mol L^{-1} luminol solution was chosen for the succeeding studies.

Considering that the luminol oxidation is favoured in alkaline medium, distinct NaOH solutions with increasing concentrations were evaluated. It was observed that the CL emission reached a maximum value for 0.2 mol L^{-1} NaOH.

3.1.2. Physical parameters

By comparing chemiluminescence intensities using reaction coils of different lengths (10–100 cm) in the presence of 1.0×10^{-5} mol L^{-1} luminol and 1.0×10^{-7} mol L^{-1} myoglobin, it was observed that the chemiluminescence intensity did not diverge significantly. Nevertheless, aiming at guaranteeing a good mixture and in order to not compromise the determination rate two 50-cm reaction coils were selected for the subsequent experiments. Flow rate was evaluated between 0.6 and 3.0 ml min^{-1}. Since no significant differences were verified in the chemiluminescence response, a flow rate of 2.0 ml min^{-1} was chosen. These results showed that reaction coil length and flow rate were not fundamental parameters affecting the intensity of the analytical signal obtained with the SIFA system, which could be probably explained by the fact that since sample and reagent solutions have no fixed physical limits any increase in the residence time would be compensated by the continuous surplus of solutions participating in the single reaction interface as it expands to the neighbouring intact (non-dispersed) zones.

Table 1
Results obtained in the evaluation of the interfering effect of selected compounds by using a 50 mmol L^{-1} mannitol solution

Added1species	Tolerance limit
Cl^-, NO_3^-, Ac^-, I^-, SO_4^{2-}, PO_4^{2-}	100[a]
Na^+, Ni^{2+}, Zn^{2+}, Cr^{3+}, Mg^{2+}, Ca^{2+}, Cu^{2+}	100
Sorbitol, glucose, borate, oxalate, malate, ureia	100
Uric acid, NH_4^+, BrO_3^-	10
Fe^{3+}	1

Data refer to the concentration ratio (expressed in mol L^{-1}) between the interfering specie and the analyte.
[a] Maximum tested concentration ratio.

3.2. Interferences

In order to assess the selectivity of the developed approach it was applied in the analysis of a 50 mmol L^{-1} mannitol solution containing increasing amounts of several species that are either used as excipients in pharmaceutical formulations or appeared in the composition of human urine samples. A chemical specie was considered as non-interfering if the analytical signal variation was lower than 5% regarding the one obtained in its absence. The obtained results (Table 1) showed no significant interfering effect for the majority of the tested compounds with a tolerable molar concentration ratio of about 100, except for iron(III) which interferes at concentration values similar to those of mannitol. These results confirmed that the proposed methodology can be applied in the analysis of mannitol in both pharmaceutical formulations and urine.

3.3. Application to pharmaceutical formulations and biological samples

After system optimisation and by using the analytical conditions exhibited in Table 2, a linear working range for mannitol concentrations between 25 mmol L^{-1} and 1 mol L^{-1}, was obtained (Fig. 2). The calibration curve was expressed by the equation:

$$I = -15.952C + 92.224$$

where I represents the relative chemiluminescence intensity and C is the logarithm of mannitol concentration (expressed in mol L^{-1}). The correlation coefficient was 0.9997.

In order to evaluate the applicability and the accuracy of the developed methodology in the analysis of real samples, it was

Table 2
Parameters evaluated during the optimisation of the SIFA system performance and most favourable values selected for its operation

Parameter	Evaluated range	Selected values
Reactor length (cm)	10–100	50
Flow rate (mL min^{-1})	0.6–3	2.0
Myoglobin concentration ($\times 10^{-7}$ mol L^{-1})	1.0–6.0	4.0
Luminol concentration ($\times 10^{-5}$ mol L^{-1})	0.1–5.0	1.0
Mannitol concentration (mol L^{-1})	1×10^{-3}–1	25×10^{-3}–1

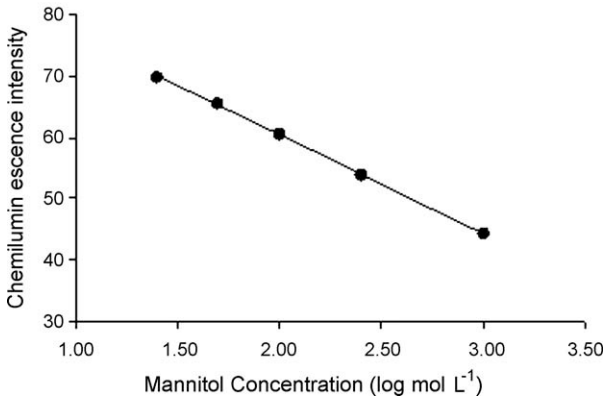

Fig. 2. Results obtained in the calibration of the system. Mannitol standards concentrations between 25 mmol L^{-1} and 1 mol L^{-1}.

Table 3
Results obtained by using the SIFA methodology (C_{SIFA}) and the reference method (C_{Ref})

Sample	C_{SIFA} (% m/V)	C_{Ref} (% m/V)	R.D. (%)[a]
Manitosteril 10%	10.2 ± 0.1	9.8 ± 0.003	3.8
Osmofundina 17.5%	17.9 ± 0.1	18.7 ± 0.034	−4.2
Manitol 20%	20.9 ± 2.4	20.0 ± 0.064	4.6

Each value corresponds to the mean ± standard deviation.
[a] Relative deviation (expressed in percentage) of the developed SIFA methodology regarding the reference procedure.

applied to the determination of mannitol in commercially available pharmaceutical formulations. The obtained results (Table 3) exhibited a good agreement between the results furnished by both methods, with relative deviations (expressed in percentage) lower than 4.6%. Furthermore, the repeatability was good, with a relative standard deviation lower than 4.7% ($n = 3$), and the determination rate was about 60 h^{-1}. For comparison purposes a paired t-test was also performed on the data obtained by the proposed method and by the reference method. A t value of 0.39 was obtained, which was lower than the tabulated t value = 4.30 ($P = 0.05$, d.f = 2) indicating no significant differences for the mean concentrations obtained by both methods. When applied in the analysis of human urine samples the developed chemiluminometric SIFA methodology showed as well a good performance, with recovery values (expressed in percentage of the added amount) ranging from 94.9 to 105.3% (Table 4), indicating that the proposed analytical approach is suitable for the monitoring of mannitol in these biological samples.

The developed flow systems exhibited good stability (no baseline drift was observed) and robustness. The utilisation of solenoid micro-pumps guaranteed a good operational simplicity, and enhanced sample/reagent mixing ability, an important feature for carrying out chemiluminescence measurements that assure as well low solutions consumptions and the minimisation of hazardous waste generation. When compared with more conventional CL flow methodologies SIFA systems revealed a simpler configuration and control while the occurrence of operational errors is minimised because they do not require the reproducible insertion of well-defined sample and reagent volumes.

Table 4
Results obtained in the determination of mannitol in spiked human urine samples (amount added: 100 mmol L^{-1}) using the SIFA methodology

Urine Sample	Original amount (mmol L^{-1})	Amount found (mmol L^{-1})	Recovery (%)
1	30.6	133.7	103.1
2	40.7	135.6	94.9
3	545.2	650.5	105.3

Each value corresponds to the average of three determinations.

4. Conclusion

The proposed single interface system allowed fast and reliable determination of mannitol in pharmaceutical formulations and in urine. For moreover, the proposed method has an innovator characteristic since, contrary to the existing methods, it uses mannitol radical scavenger property for its determination, which improves selectivity.

The developed system presented an acceptable relative standard deviation that demonstrates a good repeatability. This feature is certainly a consequence of the particular characteristic of SIFA systems that is the establishment of the reaction zone without using definite sample and reagent volumes. When compared to the reference procedure, relative deviations were inferior to 4.6%. The accuracy of the proposed methods face to the reference method was confirmed by paired t-test for pharmaceutical formulations, as well as, the obtained recoveries in urine spiked samples.

The proposed method requires no sample pretreatment, provides a wide working concentration range and is more versatile than most of the reported methods. Furthermore, single interface flow system presents a simpler optimisation than other flow techniques.

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CAPÍTULO 4

Avaliação da utilização de aceitadores-π para a determinação de hormonas da tiróide usando um sistema de fluxo com interface única

As hormonas da tiróide, levotiroxina (T_4) e liotironina (T_3), apresentam um papel muito importante no normal desenvolvimento do sistema nervoso central de lactentes, no crescimento ósseo de crianças, assim como, no funcionamento normal de vários órgãos em adultos. Uma produção deficiente ou excessiva destas hormonas resulta em patologias conhecidas como hipotiroidismo e hipertiroidismo, respectivamente. Para o tratamento do hipotiroidismo são utilizadas preparações farmacêuticas destas hormonas da tiróide.

Assim, neste trabalho foi desenvolvido um sistema SIFA para a determinação espectrofotométrica destas hormonas em formulações farmacêuticas.

O método proposto explora, pela primeira vez, a formação de complexos de transferência de carga entre as hormonas levotiroxina e liotironina, actuando como dadores de electrões, e aceitadores-π, como o ácido cloroanílico e a 2,3-dicloro-5,6-diciano-p-benzoquinona, respectivamente.

O sistema SIFA implementado permitiu a determinação de levotiroxina e liotironina com concentrações compreendidas entre $5{,}0\times10^{-5}$ e $2{,}5\times10^{-4}$ mol L^{-1}, e $1{,}0\times10^{-5}$ e $1{,}0\times10^{-4}$ mol L^{-1}, respectivamente, e com um ritmo de determinação de 26 h^{-1}, para as duas hormonas.

Os resultados obtidos para as formulações farmacêuticas foram estatisticamente comparáveis à quantidade de hormona declarada, com desvios relativos inferiores a 2,1%. A exactidão foi igualmente confirmada através da realização de ensaios de recuperação, que forneceram valores de recuperação satisfatórios entre 96,3% e 103,7% para a levotiroxina e 100,1% para a liotironina.

Para além disso, a versatilidade dos sistemas SIFA foi demonstrada ao permitir a implementação do método de Job ou método das variações contínuas para o estabelecimento da estequiometria dos complexos de transferência de carga formados nas reacções propostas.

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Exploiting π-acceptors for the determination of thyroid hormones (T3 and T4) using a single interface flow system

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ABSTRACT

A fully automated methodology was developed for the determination of the thyroid hormones levothyroxine (T4) and liothyronine (T3). The proposed method exploits the formation of highly coloured charge–transfer (CT) complexes between these compounds, acting as electron donors, and π-acceptors such as chloranilic acid (CLA) and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ). For automation of the analytical procedure a simple, fast and versatile single interface flow system (SIFA) was implemented guaranteeing a simplified performance optimisation, low maintenance and a cost-effective operation. Moreover, the single reaction interface assured a convenient and straightforward approach for implementing Job's method of continuous variations used to establish the stoichiometry of the formed CT complexes.

Linear calibration plots for levothyroxine and liothyronine concentrations ranging from 5.0×10^{-5} to 2.5×10^{-4} mol L^{-1} and 1.0×10^{-5} to 1.0×10^{-4} mol L^{-1}, respectively, were obtained, with good precision (R.S.D. <4.6% and <3.9%) and with a determination frequency of 26 h^{-1} for both drugs. The results obtained for pharmaceutical formulations were statistically comparable to the declared hormone amount with relative deviations lower than 2.1%. The accuracy was confirmed by carrying out recovery studies, which furnished recovery values ranging from 96.3% to 103.7% for levothyroxine and 100.1% for liothyronine.

1. Introduction

Charge–transfer (CT) reactions, which involved the formation of intensely coloured complexes between electron donors with a sufficiently low ionization potential, and acceptors exhibiting a sufficiently high electron affinity [1], have been extensively studied and applied in distinct analytical circumstances including the spectrophotometric determination of a variety of pharmaceutical products. Some π-acceptors such as chloranilic acid (CLA), 2,6-dichloroquinone-4-chloramide (DCQ), 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) and 7,7,8,8-tetracyano-*p*-quinodimethane (TCNQ) are known to yield CT complexes and radical ions with a variety of electron donors [2].

Thyroid hormones (3,5,3′,5′-tetraiodothyronine, T4 and 3,5,3′-triiodothyronine, T3) are compounds with a noteworthy biological relevance as they play a critical role in the normal development of the central nervous system in infants, skeletal growth and maturation in children, as well as in the normal functioning of multiple organ systems in adults [3]. A deficient or excessive production of thyroid hormones may result in clinical disorders in man known as hypothyroidism and hyperthyroidism, respectively. For the treatment of hypothyroidism, thyroid hormone pharmaceutical preparations are used.

Several analytical methods relying on immunoassays [4,5], electrochemistry [6–8], chromatography [9–14], spectrophotometry [15,16], fluorescence [17] and chemiluminescence [18] have been reported for the determination of thyroid hormones in body fluids and pharmaceuticals. Although some of them are fairly selective they also present important shortcomings, *e.g.* utilization of expensive instrumentation, complex operation and maintenance, narrow working dynamic range, use of hazardous radioactive materials or toxic solvents, etc. In addition, low sample throughput has been often noted, as they may require several minutes per assay cycle for sample incubation or may involve lengthy procedures such as, *e.g.* those requiring column preparation or preparation of working electrodes.

Flow injection (FIA) and sequential injection (SIA) analytical procedures have already been reported for the determination of thyroid hormones with amperometric [8] and UV [15] detection, respectively. The main advantages of flow-based methods include robustness, simplicity and low cost of instrumentation, rapidity of analysis and excellent precision and accuracy.

The recently proposed single interface flow systems (SIFA) [19] presents some additional advantages in relation to FIA and SIA

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systems that rely on the premise that well-defined and compelling sample and reagent volumes no longer have to be optimised because reaction development depends exclusively on the establishment, during system operation, of an unique reaction interface where mutual sample and reagent interpenetration occurs, which facilitates system configuration thus enhancing simplicity and operational versatility.

In this work the formation of CT complexes between levothyroxine and liothyronine, as electron donors, and π-acceptors such as CLA, DCQ, DDQ and TCNQ has been for the first time investigated. The analytical potential of SIFA flow systems was exploited for implementation of fully automated flow-based procedures for the spectrophotometric monitoring of these thyroid hormones in pharmaceutical formulations. The versatility of SIFA also enabled the on-line implementation of the Job's method of continuous variations aiming at establishing the stoichiometry of the formed CT coloured complex.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with water from a Milli-Q system (conductivity < 0.1 $\mu S\,cm^{-1}$) and chemicals of analytical reagent grade quality. Reagents were then not subject to any further purification.

3,5,3′,5′-tetraiodothyronine (T4, levothyroxine) and 3,5,3′-triiodothyronine (T3, liothyronine) were purchased from Sigma (St. Louis, MO, USA). A 1.0×10^{-3} mol L^{-1} T4 solution was daily prepared by dissolving 22.2 mg in 25 mL of absolute ethanol (Panreac, Barcelona, Spain) and ultrasonicating for 30 s. Working standard solutions were prepared by suitable dilutions with absolute ethanol. A 1.0×10^{-4} mol L^{-1} T3 solution was daily prepared by dissolving 3.36 mg in 0.120 mL of a 0.1 mol L^{-1} NaOH solution, ultrasonicating for 30 s; this solution was diluted up to 50 mL with absolute ethanol. Working standard solutions were prepared by suitable dilutions with absolute ethanol.

CLA (BDH, Poole, UK), DCQ (Merck, Darmstadt, Germany), DDQ (Sigma, Steinheim, Germany) and TCNQ (Fluka, Austria) were used without further purification. The 1.0×10^{-3} mol L^{-1} CLA and DDQ solutions were daily prepared by dissolving 20.9 and 22.7 mg, respectively, in 100 mL of absolute ethanol.

2.2. Sample treatment

Commercial tablets with nominal contents of 25, 50 and 100 μg T4 and 25 μg T3 were analysed. To this end, twenty tablets were weighted and finely grounded. An accurately weighed powder equivalent to about 100 μg T4 or 25 μg T3 was dissolved in absolute ethanol, subject to ultrasonication for 30 s, and then filtered through a 0.20 μm membrane filter.

2.3. Apparatus

The SIFA system comprised two automatic burettes model Micro BU 2031 (Crison Instruments, Allelam, Spain) equipped with 5 mL syringes, two 161 T 031 (NResearch, West Caldwell, USA) two-way solenoid valves, a Jenway 6305 spectrophotometer (Jenway, Dunmow, UK) equipped with a 80 μL internal volume flow cell (10 mm optical path) and reaction coils made of PTFE tubing (0.8 mm i.d.). Home made end-fittings and connectors were also used.

A Pentium-I-based computer was used for system control and for data acquisition and treatment; software was developed in Microsoft Quick-Basic 4.5. The computer was equipped with a PC-LABCard model PCL-711B (Advantech, Taipei, Taiwan) interface card. The automatic burettes were connected to a serial port (RS-232C). A homemade power drive based on the ULN2003 chip or a CoolDrive (NResearch Inc., West Caldwell, USA) was used to operate the solenoid valves.

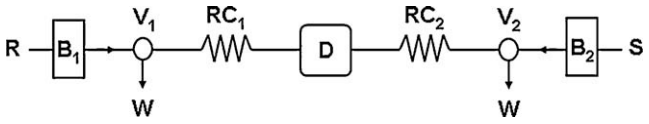

Fig. 1. Single interface flow manifold for the determination of T3 and T4. B_1 and B_2: automatic burettes; V_1 and V_2: solenoid valves; RC_1 and RC_2: reaction coils (65 cm); D: spectrophotometric detector; W: waste; R: reagent, π-acceptor; S: sample or standard.

Analytical signals were also recorded in paper using a Kipp & Zonen (Delft, The Netherlands) BD 111 strip chart recorder.

Spectrophotometric measurements were carried out in a UV–vis Spectrometer model Lambda 45 (Perkin-Elmer Instruments Inc., Norwalk, USA).

2.4. Single interface flow manifold and procedure

The flow manifold (Fig. 1) comprised two automatic burettes (B_1 and B_2) for inserting and propelling the sample and reagent solutions, and two two-way (normally closed) solenoid valves (V_1 and V_2) for directing the flowing streams. The reactions coils, identically lengthened, were placed on both sides of the detector.

The analytical cycle was started by establishing baseline, which was accomplished with the π-acceptor solution. To this end, V_1 was open and B_1 was actuated for propelling the reagent through the reactors as well as through the detector. After reaching V_2 these solutions were discarded. Subsequently, V_2 was opened, B_1 was switched off and the sample solution was inserted into the analytical path by means of actuation of B_2 establishing the single sample/reagent interface that was propelled backwards in order to reach V_1. The reaction products formed as a consequence of the mutual sample/π-acceptor intermingle produced an analytical signal, which was recorded as a peak when the reaction interface passed through the spectrophotometric flow cell. The absorbance of the T4–CLA complex was measured at 538 nm whereas the T3–DDQ complex was monitored at 460 nm.

3. Results and discussion

3.1. Reaction mechanism and spectral characterization

Levothyroxine and liothyronine present high electron density sites, so they may act as powerful electron donors. In the presence of a polar solvent like ethanol, these substances exhibit a maximum absorption band at 300 nm. Upon addition of different π-acceptors to the drug solution, namely CLA, DCQ, DDQ and TCNQ, a bathochromic shift was observed, which could be attributed to the formation of CT complexes. Interaction of T4 with CLA led to a strong absorption band at 538 nm whereas the T3–DDQ complex exhibited maximum absorption at 460 nm (Fig. 2).

The selection of the most suitable solvent for reaction implementation is a very important aspect affecting not only the sample solubility but also the electron transfer and the dissociation of the formed complex into the chromogen free radical anion. In order to select the solvent for CT complex formation, the reaction of CLA, DCQ, DDQ and TCNQ with both T3 and T4 was carried out in different solvents, such as acetonitrile, methanol and ethanol. Acetonitrile did not dissolve both compounds, T4 exhibit similar solubility in methanol and ethanol and T3 was fairly soluble in methanol but only slightly soluble in ethanol. For this reason, T3 was initially solubilised in a minute quantity of sodium hydroxide

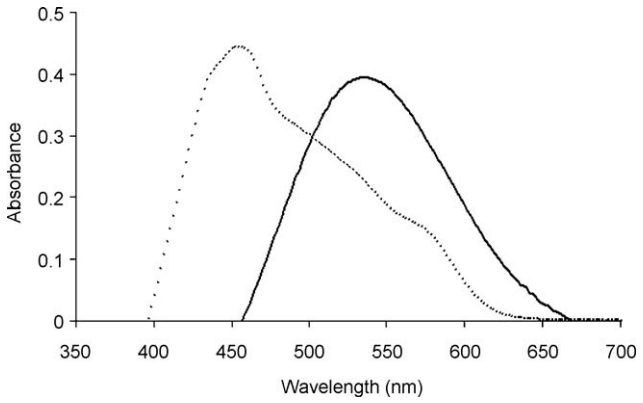

Fig. 2. Absorption spectra of T4–CLA (solid line) and T3–DDQ (dashed line) complexes in ethanol.

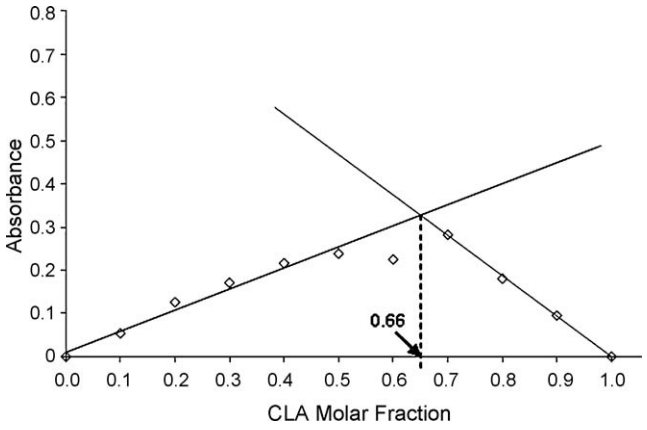

Fig. 3. Continuous variation plot for the reaction of T4 and CLA.

and then diluted with ethanol. When comparing the performance of methanol with ethanol, the later was considered the most adequate solvent for reaction development because it provided an excellent solvating power for CLA, DCQ, DDQ and TCNQ, and enabled the highest yield of the radical anion. As ethanol is a more polar solvent complete transfer of charge from donor to acceptor thus formation of radical anion as the predominant chromogen was more easily accomplished.

The π-acceptor was selected by taking into consideration the highest colour intensity of the formed complex for the same thyroid hormone concentration. For the T4 determination, CLA was chosen as the ideal π-acceptor, whereas DDQ was elected for the T3 determination. These reagents formed the complexes with the highest molar absorptivities and provided fast reaction kinetics even at room temperature.

As it was previously referred, upon reaction of CLA with T4, in ethanol medium, a characteristic large wavelength absorption band at 538 nm was noted, emphasizing the formation of a CT complex through the interaction of CLA as π-acceptor and the studied substance as *n*-donor followed by the formation of radical anion according to the following scheme:

$$\overset{D}{\text{donor}} + \overset{A}{\text{acceptor}} \leftrightarrow \overset{(D-A)}{n-\pi\,\text{complex}} \leftrightarrow \overset{A^{\bullet-}+D^{\bullet-}}{\text{radical ions}}$$

The dissociation of the complex was fostered in view of the ionizing potential of ethanol, which promoted complete electron transfer from the donor to the acceptor, resulting in the formation of the CLA radical anion as the major chromogen. The mechanism for the liothyronine–DDQ complex was similar.

3.2. Chemical parameters

Influence of CLA and DDQ concentration was investigated for concentrations ranging from 1.0×10^{-4} to 5.0×10^{-3} mol L^{-1} and from 2.5×10^{-5} to 1.0×10^{-3} mol L^{-1}, respectively. In both cases the obtained analytical signals showed a pronounced increase up to CLA and DDQ concentration values of 1.0×10^{-3} mol L^{-1} approaching stabilisation for higher values. Therefore, 1.0×10^{-3} mol L^{-1} CLA and DDQ solutions were used in the subsequent experiments.

3.3. Single interface flow methodology

In the development of the SIFA methodology several experiments were carried out in order to improve system performance, namely in terms of sensitivity, accuracy, precision and sampling rate. These features influenced the choices made during the optimisation of the systems, which was carried out using the univariate method. Since no well-defined sample or reagent volumes were used, these parameters were not subject to evaluation, thus simplifying the optimisation process.

The length and configuration of the reaction coils were assessed, for each of the hormones, by using 1.0×10^{-4} mol L^{-1} T4 and 5.0×10^{-4} mol L^{-1} CLA, and 2.5×10^{-4} mol L^{-1} T3 and 2.5×10^{-4} mol L^{-1} DDQ, respectively. The evaluation of the analytical signals obtained with reaction coils ranging from 15 to 115 cm revealed that the analytical signal almost double in both cases up to a coil length of 65 cm, beyond which it approached stabilisation. The shape of the reactor was also assessed by evaluating 65 cm reactors with straight and serpentine configurations. The serpentine configuration provided higher analytical signals probably due to an improved radial mass transport that assures a good sample/reagent mixing while minimising sample broadening and dispersion. Consequently, 65 cm serpentine reactors were selected for further experiments.

Influence of flow rate of the π-acceptor solution used to propel the reaction interface was assessed between 0.1 and 1.0 mL min^{-1}. Increasing flow rate increased the magnitude of the analytical signal up to 0.3 mL min^{-1} and then a slight decrease was observed. A 0.3 mL min^{-1} flow rate was then selected.

After optimization, the SIFA system was used to implement the Job's method of continuous variations in order to estimate the stoichiometry of the formed CT complexes by evaluating the molar ratio of the reactants (drug:π-acceptor) in the formed CT complexes. The results allowed one to infer molar ratios of 1:2 for T4–CLA (Fig. 3) and of 1:3 for T3–DDQ.

3.4. Interferences

After system dimensioning, the interfering effects of the excipients present in the pharmaceutical formulations, such as talc, starch, lactose, sodium citrate and magnesium stearate, were evaluated. A potential interfering species was considered as non-interfering if the analytical signal variation was lower than 3% in relation to the one obtained in its absence. It was found that up to a 100-fold excipient/drug molar ratio (maximum tested value) no interfering effect was observed. These results confirmed the suitability of the proposed method for the determination of T4 and T3 in pharmaceutical formulations.

3.5. Application to pharmaceutical formulations

Under the analytical conditions exhibited in Table 1, linear working concentration ranges between 5.0×10^{-5} to 2.5×10^{-4} mol L^{-1} and 1.0×10^{-5} to 1.0×10^{-4} mol L^{-1} were obtained for T4 and T3,

Table 1
Range of values used in dimensioning the SIFA system, and selected operating conditions for the determination of T4 and T3.

Parameter	Range	Chosen value
Reactor length (cm)	15–115	65
Flow rate (mL min^{-1})	0.1–1.0	0.3
CLA concentration (mol L^{-1})	1.0×10^{-4} to 5.0×10^{-3}	1.0×10^{-3}
T4 concentration (mol L^{-1})	1.0×10^{-5} to 1.0×10^{-3}	5.0×10^{-5} to 2.5×10^{-4}
DDQ concentration (mol L^{-1})	2.5×10^{-5} to 1.0×10^{-3}	1.0×10^{-3}
T3 concentration (mol L^{-1})	1.0×10^{-6} to 1.0×10^{-4}	1.0×10^{-5} to 1.0×10^{-4}

Table 2
Results obtained by SIFA methodology (C_{SIFA}) and declared amounts.

Sample	Declared amount (μg)	C_{SIFA}[a] (μg)	RD (%)
Letequatro (T4)	100	101.4 ± 1.5	1.4
Letter 20 pills (T4)	100	98.4 ± 2.1	−1.6
Letter 60 pills (T4)	100	98.7 ± 1.9	−1.3
Thyrax 25 (T4)	25	24.5 ± 0.1	−2.1
Thyrax 100 (T4)	100	101.4 ± 0.7	1.4
Eutirox 25 (T4)	25	24.8 ± 0.4	−0.8
Eutirox 50 (T4)	50	50.6 ± 0.3	1.2
Eutirox 100 (T4)	100	99.2 ± 3.1	−0.8
Neo-tiroimade (T3)	25	24.9 ± 0.5	0.5

[a]Mean and deviations based on three replications.

respectively. The analytical curves were typically expressed as

$$T4 : A = 1045C + 0.033$$

$$T3 : A = 4025.6C + 0.0163$$

where A is the absorbance and C is the T4 or T3 molar concentration (expressed in mol L^{-1}). The correlation coefficients were 0.9992 and 0.9990 for T4 and T3, respectively.

In order to evaluate the applicability and the accuracy of the proposed method in the analysis of real samples, it was applied to commercially available pharmaceutical formulations. The results (Table 2) were in a fairly good agreement with the amount labelled, with relative deviations between −2.1% and 1.4%. Furthermore, the repeatability was good, with a relative standard deviation lower than 4.6% and 3.9% ($n = 3$) for T4 and T3, respectively, and the sampling rate was about 26 h^{-1} for both analyses.

The accuracy was assessed by spiking levothyroxine and liothyronine formulations with a known concentration of T4 or T3 standard (100 and 25 μg, respectively). Recoveries ranging from 96.3% to 103.7% were found for T4 and a recovery of 100.1% was found for T3.

4. Conclusions

Taking into consideration the chemical structure of levothyroxine and liothyronine, which could act as electron donors, the first time exploitation of π-acceptors as a means to form CT sample/reagent interactions assured a good selectivity and sensitivity as well as an expeditious, simple and accessible chemical pathway for the spectrophotometric determination of these hormones.

The proposed SIFA system allowed fast and reliable analyses of pharmaceutical formulations, with high operational simplicity, good system ruggedness and stability and low maintenance. The SIFA system yields high reproducible and reliable results as confirmed by the low relative deviations between the declared and found amounts as well as the good recovery data obtained in the selectivity studies. Moreover, the single reaction interface assured a convenient and straightforward approach for implementing Job's method of continuous variations used to establish the stoichiometry of the CT complexes formed between T4 or T3 and the π-acceptors (CLA and DDQ, respectively).

When compared with most of the reported methods for the determination of thyroid hormones, the proposed method is simple, versatile and cost-effective. Furthermore, SIFA systems are easier optimized thus potentially more robust than other flow-based analytical procedures. These features make the developed SIFA system suitable for the routine quality control of these drugs in raw material and tablets without interference from the excipients present in the formulations.

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CAPÍTULO 5

Desenvolvimento de um sistema de fluxo com interface única com avaliação da exactidão

Os sistemas SIFA apresentam algumas vantagens quando comparados com outros sistemas de fluxo, como por exemplo, uma configuração mais simples, uma forma de operar e controlar mais fácil e uma rotina de optimização menos exigente. Para além disso, o estabelecimento da zona de reacção, que se baseia estritamente na penetração de zonas adjacentes, pode ser explorada para realizar vários esquemas reaccionais de forma sequencial, permitindo a aquisição de informação suplementar sobre os analitos que estão a ser determinados.

Neste contexto, estratégias de avaliação da exactidão podem ser implementadas. Para tal, a amostra é processada por dois métodos analíticos *quasi*-independentes e o resultado final é calculado após considerar os resultados obtidos por estes dois métodos. Desta forma, obtêm-se resultados mais precisos e exactos.

Para demonstrar a viabilidade desta estratégia, um sistema SIFA com detecção espectrofotométrica foi desenvolvido para a determinação de lansoprazol em formulações farmacêuticas. Assim, duas interfaces de reacção, obtidas através da reacção com dois distintos aceitadores-π, ácido cloroanílico e a 2,3-dicloro-5,6-diciano-p-benzoquinona, foram implementadas. A análise da amostra no sistema SIFA proposto originava, desta forma, dois sinais analíticos resultantes de medições a diferentes comprimentos de onda, sendo utilizados para calcular a concentração do analito na amostra.

Os resultados obtidos utilizando o sistema SIFA desenvolvido foram comparados aos fornecidos pelo método de referência, apresentando desvios relativos inferiores a 2,7%. Para a avaliação da exactidão foi também aplicado o teste *t* de Student emparelhado que permitiu concluir que não havia diferenças estatisticamente significativas entre os resultados obtidos pelos dois métodos. O ritmo de determinação obtido foi de 30 h^{-1}.

Para além de se expandir o conceito de SIFA, estabelecendo duas interfaces de reacção como forma de mostrar a viabilidade destes sistemas para a implementação da avaliação da exactidão, convém mencionar que neste trabalho, a formação de complexos de transferência de carga entre o lansoprazol e os aceitadores-π foi explorada, pela primeira vez, para a determinação deste analito em formulações farmacêuticas.

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Single interface flow analysis with accuracy assessment

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ABSTRACT

Single interface flow systems (SIFA) present some noteworthy advantages when compared to other flow systems, such as a simpler configuration, a more straightforward operation and control and an undemanding optimisation routine. Moreover, the plain reaction zone establishment, which relies strictly on the mutual inter-dispersion of the adjoining solutions, could be exploited to set up multiple sequential reaction schemes providing supplementary information regarding the species under determination. In this context, strategies for accuracy assessment could be favourably implemented. To this end, the sample could be processed by two *quasi*-independent analytical methods and the final result would be calculated after considering the two different methods. Intrinsically more precise and accurate results would be then gathered.

In order to demonstrate the feasibility of the approach, a SIFA system with spectrophotometric detection was designed for the determination of lansoprazole in pharmaceutical formulations. Two reaction interfaces with two distinct π-acceptors, chloranilic acid (CLA) and 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) were implemented.

Linear working concentration ranges between 2.71×10^{-4} to 8.12×10^{-4} mol L^{-1} and 2.17×10^{-4} to 8.12×10^{-4} mol L^{-1} were obtained for DDQ and CLA methods, respectively. When compared with the results furnished by the reference procedure, the results showed relative deviations lower than 2.7%. Furthermore, the repeatability was good, with r.s.d. lower than 3.8% and 4.7% for DDQ and CLA methods, respectively. Determination rate was about 30 h^{-1}.

1. Introduction

Analytical flow systems with accuracy assessment are valuable resources in routine laboratories as they can provide a reinforced guarantee regarding the quality of the obtained results, as well as additional information on the specificities of a particular set of samples, mainly in terms of matrix effects, assuring a more appropriate management of the routine process control. The concept of accuracy assessment relies on processing the sample simultaneously by two *quasi*-independent analytical methods thus enabling intrinsically more accurate results given that the final analyte concentration is calculated by taking into consideration the results provided by the two methods.

Quality control of pharmaceutical formulations, either during the production stage, as final products or even for screening counterfeit medicine is a crucial concern demanding the selection of accurate, robust and expeditious analytical techniques yielding reliable results.

As emphasised by Oliveira et al. [1], one of the main advantages of automated flow-based analytical methodologies is the high sampling throughput they usually provided, which is mainly supported by the efficient management of all solutions involved, the plain transport of the reaction zone towards detection and the unneed for measurements under equilibrium conditions.

This evident advantage of flow analysis has not been fully exploited in most of the analytical circumstances, even when a bulky sample analysis is required, due to operational limitations not directly related with the determination itself but mainly with sample collecting and pre-treatment steps. However, when this limitation does not exist, as is the case of pharmaceutical formulations analysis, the high sample throughput provided by automated flow-based methodologies could be used to improve results quality.

The application of real time accuracy assessment in a flow analysis was initially proposed in a multi-commutation flow system [1] and subsequently in sequential injection analysis (SIA) [2–6]. While the multi-commutated flow manifold was a relatively complex set up, the SIA technique upheld a more straightforward configuration that allowed the simultaneous implementation of different analytical methods in the same manifold without the need to physically reconfigure it. The recently proposed SIFA systems [7] present some additional advantages in relation to typical flow systems, as they rely on the fact that well-defined and compelling sample and reagent volumes no longer have to be optimised because reaction development depends exclusively on the establishment of a unique reaction interface where mutual sample and reagent interpenetration occur.

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This aspect facilitates system configuration thus enhancing simplicity and operational versatility.

In this work the SIFA concept was further extended by establishing two single reaction interfaces that were applied in the spectrophotometric determination of lansoprazole in pharmaceutical formulations upon simultaneous reaction with the π-acceptors chloranilic acid (CLA) and 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ). The mean of the results obtained by the two spectrophotometric methods provided a more accurate result, showing the viability of a SIFA system with accuracy assessment. Formation of charge transfer complexes between lansoprazole, as electron donor, and π-acceptors CLA and DDQ was for the first time exploited for the spectrophotometric monitoring of this drug in pharmaceutical formulations.

Lansoprazole, 2-[[3-methyl-4-(2,2,2-trifluoroethoxy)-2-pyridinyl] methyl] sulphinylbenzimidazole, is an important proton pump inhibitor being used in the treatment of peptic ulcer disease and in other health conditions where inhibition of gastric secretions may be beneficial, such as dyspepsia, gastro-oesophageal reflux disease and Zollinger–Ellison syndrome [8].

The United States Pharmacopoeia [9] is the only reference that presents the assay of lansoprazole, describing a high performance liquid chromatographic method. Furthermore, several analytical methods relying on electrochemistry [10–12], chromatography [13–26], capillary electrophoresis [27] and spectrophotometry [28–33] have been reported for the determination of lansoprazole in biological fluids and pharmaceuticals. Even though some of them are rather selective, they also have important shortcomings such as utilisation of expensive instrumentation, complex operation and maintenance, low sample throughput as they may require several minutes per assay cycle for sample incubation or may involve lengthy procedures such as those requiring preparation of chromatographic columns or working electrodes. A flow injection (FIA) system has been also reported for the determination of lansoprazole with UV detection [34].

2. Experimental

2.1. Reagents and solutions

Lansoprazole was purchased from Sigma (St. Louis, MO, USA). A 2.71×10^{-3} mol L^{-1} stock solution was daily prepared by dissolving 25 mg in 1.0 mL of a 0.1 mol L^{-1} NaOH solution and diluting up to 25 mL with absolute ethanol (Panreac, Barcelona, Spain). Working standard solutions were prepared by suitable dilutions with absolute ethanol.

Chloranilic acid (CLA, BDH, Poole, UK) and 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ, Sigma, Steinheim, Germany) were used without further purification. The 1.0×10^{-3} mol L^{-1} CLA and DDQ solutions were daily prepared by dissolving 20.9 mg and 22.7 mg of solid, respectively, in 100 mL of absolute ethanol.

2.2. Sample preparation

Commercial capsules with nominal contents of 15 and 30 mg of lansoprazole were analysed. To this end, ten capsules were emptied and the mass of the collected contents was weighted and finely grounded. An accurately weighed powder equivalent to about 15 mg lansoprazole was dissolved in a minimum quantity of a 0.1 mol L^{-1} NaOH solution, diluted with absolute ethanol up to 25 mL, and then filtered through a 0.45 μm membrane filter. Then this solution was conveniently diluted with absolute ethanol to fit the calibration curve.

2.3. Apparatus

The single interface flow system comprised three 10-μL per stroke solenoid micro-pumps (Bio-Chem Valve Inc., Boonton, USA) and a model USB2000 UV–Vis Ocean Optics (Dunedin, USA) fibre optic wavelength scanning spectrophotometer furnished with an acrylic Z-shaped flow cell (inner volume = 10 μL, optical path = 10 mm). The reaction coil was made of 0.8 mm i.d. PTFE tubing. Homemade end-fittings and connectors were also used.

A Pentium-I based computer equipped with a model PCL-711B PC-LABCard (Advantech, Cincinnati, OH) interface card was used for system control and for data acquisition and processing; software was developed in Microsoft Quick-Basic 4.5. A CoolDrive (NResearch Inc., West Caldwell, USA) or ULN2003-based homemade power drives [35] were used to operate the solenoid micro-pumps.

2.4. Flow manifold and procedure

The flow manifold (Fig. 1) comprised three solenoid pumps (P_1, P_2 and P_3) for inserting and propelling the sample and reagent solutions. The repetitive pump on/off switching created a pulsed flowing stream in which the pulse volume corresponded to the pump stroke volume.

The analytical cycle was started by establishing a baseline, which was accomplished with the DDQ π-acceptor solution. To this end, P_1 was actuated for propelling DDQ solution through the reaction coil and the detector. Subsequently, P_2 was actuated, P_1 was switched off and the sample solution (120 pump strokes of 10 μL) was inserted into the analytical path establishing the first single interface. Next, P_3 was actuated, P_2 was switched off and the CLA solution was propelled into the detector establishing the second single interface (Fig. 2). The reaction products formed as a consequence of the mutual sample/π-acceptor intermingle produced analytical signals, which were recorded when the respective reaction interfaces passed through the spectrophotometric flow cell. The absorbance of the lansoprazole–DDQ complex was measured at 462 nm whereas the lansoprazole–CLA complex was monitored at 526 nm.

2.5. Reference method

Aiming at the evaluation of the accuracy of the results obtained with the developed procedure, lansoprazole pharmaceutical formulations were analysed according to the United States Pharmacopoeia [9], by high performance liquid chromatography.

3. Results and discussion

3.1. Chemical parameters

Influence of DDQ and CLA concentrations was investigated between 1.0×10^{-4} and 2.0×10^{-3} mol L^{-1}. In both cases the obtained analytical

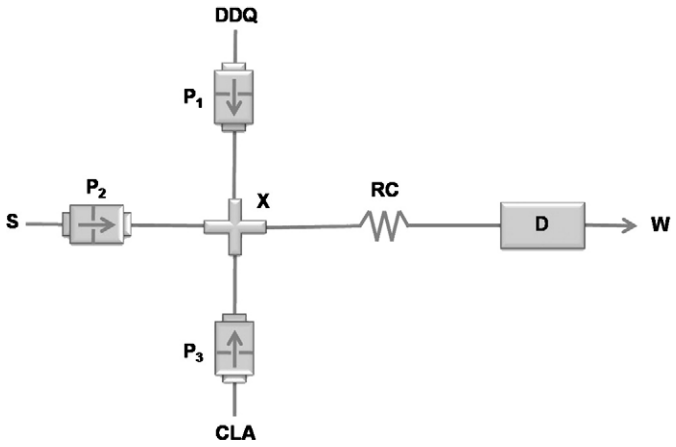

Fig. 1. Single interface flow manifold for the determination of lansoprazole. Legend: P_1, P_2, P_3: solenoid micro-pumps; DDQ: 2,3-dichloro-5,6-dicyano-p-benzoquinone; S: sample; CLA: chloranilic acid; X: confluence; RC: reaction coil; D: spectrophotometric detector; W: waste.

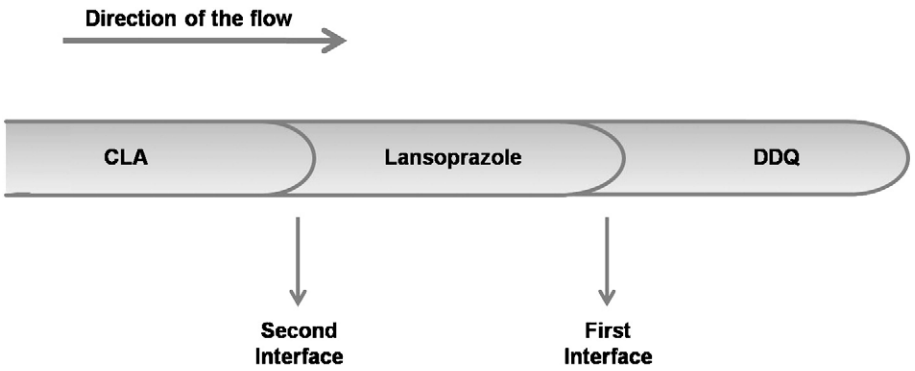

Fig. 2. Schematic presentation of the two interfaces formed in the determination of lansoprazole.

signals showed a pronounced increase up to DDQ and CLA concentration values of 1.0×10^{-3} mol L^{-1} approaching stabilisation for higher values. Therefore, 1.0×10^{-3} mol L^{-1} DDQ and CLA solutions were used in the subsequent experiments.

3.2. Physical parameters

In the development of the methodology exploiting two interfaces for implementing the accuracy assessment strategy, several experiments were carried out in order to improve system performance, namely in terms of sensitivity, accuracy, precision and sampling rate. These features influenced the choices made during the optimisation of the systems, which was carried out using the univariate method. Since no well-defined sample or reagent volumes were used, these parameters were not subjected to evaluation, thus simplifying the optimisation process.

Influence of the length of the serpentine reaction coil was assessed between 10 and 200 cm, by using 1.0×10^{-3} mol L^{-1} DDQ, 1.0×10^{-3} mol L^{-1} CLA and 2.71×10^{-4} mol L^{-1} lansoprazole. It was observed that for the lansoprazole–DDQ complex the analytical signal increased up to a coil length of 100 cm, slightly decreasing for longer ones, whereas for the lansoprazole–CLA complex the analytical signal remained almost constant above a 0.25-m coil, which provided the highest signal. These results are explained by the different kinetics of the reaction between the two π-acceptors and lansoprazole. Since CLA reacted much faster any increase in the residence time arising from the utilisation of a reaction coil longer than 0.25 m was not significant in terms of reaction development and analytical signal magnitude. On the contrary, the slower reaction kinetics of DDQ demanded the utilisation of a longer coil to attain a reaction extension similar to that obtained with CLA. A 100-cm reaction coil was then considered the most appropriate to simultaneously implement both reactions. This distinct behaviour was also evident in the assays carried out to assess the influence of flow rate (considered in terms of frequency of micro-pumps strokes). The stroke frequency used to propel the reaction interfaces was investigated between 50 and 250 strokes min^{-1} (corresponding to flow rate values between 0.5 and 2.5 mL min^{-1} for a 10 μL stroke volume). Increasing flow rate increased the magnitude of the analytical signal up to 70 strokes min^{-1} and 150 strokes min^{-1} for lansoprazole–DDQ and lansoprazole–CLA interfaces, respectively, and then a slight decrease was observed. A 110 strokes min^{-1} micro-pump frequency (1.1 mL min^{-1} flow rate) was then selected in order to satisfy the two reaction interfaces formed without compromising the determination rate.

3.3. Interferences

After system dimensioning, and in order to guarantee the applicability of the developed approach to the determination of lansoprazole in pharmaceutical formulations, the interfering effect of the excipients present in the capsules, like starch, talc, lactose, sodium citrate and magnesium stearate, was evaluated. Samples containing a fixed amount of lansoprazole and increasing concentrations of the excipient under evaluation were run. A compound was considered as non-interfering if the analytical signal variation was ±3% when compared to the analytical signal obtained in its absence. It was verified that lactose and up to a 100-fold excipient/drug molar ratio (maximum tested value) of the other potential interferents, no interfering effect was observed. These results confirmed the suitability of the proposed method for the determination of lansoprazole in pharmaceutical formulations.

3.4. Application to pharmaceutical formulations

Under the analytical conditions exhibited in Table 1, linear working concentration ranges from 2.71×10^{-4} to 8.12×10^{-4} mol L^{-1} and from 2.17×10^{-4} to 8.12×10^{-4} mol L^{-1} were obtained for DDQ and CLA methods, respectively (Fig. 3). The analytical curves were typically expressed as:

$$DDQ\ method: A = 1056.5C + 0.1663$$

$$CLA\ method: A = 305.25C + 0.0564$$

where A = absorbance and C = lansoprazole concentration expressed in mol L^{-1}. The correlation coefficients were 0.9983 and 0.9986, respectively.

In order to evaluate the applicability and the accuracy of the proposed methods in the analysis of real samples, it was applied to the commercially available lansoprazole pharmaceutical formulations (Table 2). A good agreement between the results furnished by both methods, with relative deviations lower than 2.7% was noted. Furthermore, the repeatability was good, with a relative standard deviation lower than 3.8% and 4.7% for DDQ and CLA methods, respectively, and the determination rate was about 30 h^{-1}. As it can be inferred by the results, the application of accuracy assessment in SIFA methodology provided a more precise and accurate result, based on the mean of the results obtained in the two DDQ and CLA methods.

Table 1
Range of values used in dimensioning the SIFA system, and selected operating conditions for the determination of lansoprazole.

Parameter	Range	Selected values
Reactor length (cm)	10–200	100
Flow rate (mL min^{-1})	0.5–2.5	1.1
DDQ concentration (mol L^{-1})	1.0×10^{-4}–2.0×10^{-3}	1.0×10^{-3}
CLA concentration (mol L^{-1})	1.0×10^{-4}–2.0×10^{-3}	1.0×10^{-3}
Lansoprazole with DDQ (mol L^{-1})	2.71×10^{-5}–1.08×10^{-3}	2.71×10^{-4}–8.12×10^{-4}
Lansoprazole with CLA (mol L^{-1})	2.71×10^{-5}–1.08×10^{-3}	2.17×10^{-4}–8.12×10^{-4}

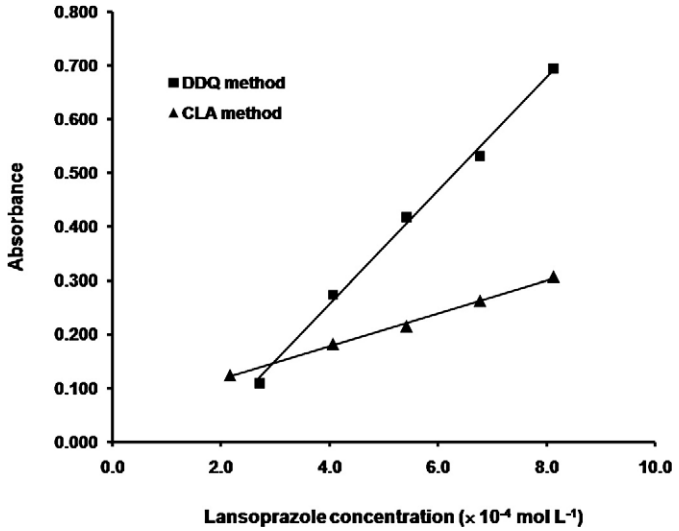

Fig. 3. Results obtained in the calibration of the system. Lansoprazole standard concentrations of 2.71×10^{-4} to 8.12×10^{-4} mol L^{-1} and 2.17×10^{-4} to 8.12×10^{-4} mol L^{-1} for DDQ and CLA methods, respectively.

For comparison purposes a paired *t*-test was also performed on the data obtained by the proposed method (averaged results) and by the reference method. A *t* value of 0.59 was obtained, which was lower than the tabulated *t* value of 4.30 ($P=0.05$, $df=2$) indicating no significant statistical differences for the mean concentrations obtained by both methods.

4. Conclusions

SIFA confirmed its versatility and analytical potential, allowing the implementation of an automated flow-based determination of lansoprazole exploiting accuracy assessment. The system combined two independent single reaction interfaces and was very simple and easily operated. Sample processing in the developed SIFA system generated two analytical signals from measurements at different wavelengths that were used to calculate the sample concentration. Accordingly, the application of accuracy assessment in the proposed SIFA system permitted the acquisition of more precise and accurate results and showed also some noteworthy advantages being simpler, less time-consuming and less laborious than the reference method.

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Table 2
Results obtained for lansoprazole in pharmaceutical formulations by using the reference method and by SIFA system with DDQ and CLA methods.

Sample	Reference method	DDQ method[a]	RD[b] (%)	CLA method[a]	RD[b] (%)	Average results	RD[b] (%)
Lansoprazol 15 mg — Medley	14.70 ± 0.10	14.6 ± 0.3	0.6	14.5 ± 0.3	1.5	14.5	1.1
Lansoprazol 30 mg — Medley	30.64 ± 0.03	31.5 ± 1.2	−2.7	29.9 ± 1.4	2.3	30.7	−0.2
Lanz 15 mg — EMS	14.65 ± 0.02	14.4 ± 0.4	1.4	14.8 ± 0.7	−1.1	14.6	0.2

[a] Estimated means and standard deviations based on three replications.
[b] Relative deviation (expressed in percentage) to the reference method.

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CAPÍTULO 6

Extracção líquido-líquido em análise em fluxo: uma revisão crítica

Desenvolvimento de um sistema de fluxo com interface única para extracção aquosa bifásica

Os processos extractivos líquido-líquido são um tipo de processamento de amostra relativamente pouco explorado em análise em fluxo. Apesar disso, comparando com os métodos manuais, a extracção líquido-líquido realizada num sistema de fluxo contribui não só para a redução do consumo de solventes orgânicos, reagentes e amostras, originando uma análise menos dispendiosa, como também assegura uma diminuição dos efluentes gerados e dos riscos de exposição para o operador.

Embora apresente vantagens notórias, o facto destes processos extractivos não serem amplamente explorados em sistemas de fluxo pode ser explicado pela dificuldade associada à implementação e posterior separação, em fluxo, de interfaces envolvendo duas fases imiscíveis (convencionalmente uma fase orgânica e uma fase aquosa). Esta dificuldade é reforçada pela toxicidade e volatilidade de grande parte dos solventes orgânicos utilizados e pela sua agressividade relativamente aos materiais usados na concepção das montagens de fluxo.

Neste âmbito, foi inicialmente elaborado um artigo de revisão visando descrever as diferentes estratégias desenvolvidas para a realização de extracções líquido-líquido em análise em fluxo, analisando as principais limitações de cada abordagem e a forma como os autores de esforçaram, ao longo dos anos, no sentido de as ultrapassar.

Através deste artigo de revisão foi possível adquirir uma visão mais ampla sobre este tema e desenvolver um trabalho subsequente que explorou, pela primeira vez, a realização de uma extracção aquosa bifásica em análise em fluxo. Este tipo de extracção destaca-se pelo facto de ser bastante atractivo em termos ambientais, na medida em que se baseia na utilização de duas fases imiscíveis que são intrinsecamente aquosas. Assim, os solventes orgânicos das extracções líquido-líquido convencionais não são mais usados, sendo substituídos por solventes que não são tóxicos, nem inflamáveis, nem voláteis.

Desta forma, verificando a aplicabilidade deste tipo de extracção em análise em fluxo, desenvolveu-se um sistema SIFA para a pré-concentração de chumbo. Após a extracção, o chumbo reagia com ácido 8-hidroxiquinolina-5-sulfónico, cujo produto de reacção era determinado através de um detector fluorimétrico incluído na montagem analítica. Assim, a interface única estabelecida foi utilizada quer como interface de extracção, quer como interface de reacção. Este trabalho permitiu demonstrar a versatilidade e o potencial analítico dos sistemas SIFA, representando um ponto de partida para novas tendências e futuras aplicações e desenvolvimentos.

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Review

Liquid–liquid extraction in flow analysis: A critical review

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ABSTRACT

Liquid–liquid extractions (LLE) are a common sample pre-treatment in many analytical applications. This review aims at providing a critical overview of the distinct automated continuous flow-based approaches that were developed for liquid–liquid extraction with the purpose of pre-concentration and/or separation of multiple analytes, such as ultra-trace metal and metalloid species, phenolic compounds, surfactants, pharmaceuticals, etc., hyphenated with many detection technique such as UV/vis spectrophotometry, atomic spectrometric detection systems and luminescent detectors, including distinct extraction strategies and applications like single and multiple extraction schemes, wetting film extraction, supported liquid membrane extraction, back extraction, closed-loop systems and the utilisation of zone sampling, chromatomembranes and iterative reversal techniques. The analytical performance of the developed flow-based LLE methods and the influence of flow manifold components such as the segmenter, extraction coil and phase separator, is emphasised and object of discussion. An overall presentation of each system components, selectivity, advantages and shortcomings is carried out and exemplified with selected applications.

Contents

1. Introduction

Despite the great diversity of analytical techniques able to provide enhanced selectivity and sensitivity, solution handling is one of the most frequently performed laboratory tasks, constituting too often a limiting step for attaining the required analytical efficiency.

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During the last decades the important role played by flow-based techniques for automation, simplified optimization and miniaturization of solution handling in sample pre-treatment has been well demonstrated. The advantageous features exhibited by these techniques, which include the minimization of both sample and reagent consumption, risk of sample contamination and operator's intervention as well as an enhanced sampling throughput and the possibility of coupling with distinct detection techniques had a deep influence in the development of new analytical approaches and in the inception of new trends in terms of analytical system modularity, flexibility and versatility.

One of the most common sample pre-treatments in an analytical process involve liquid–liquid extractions (LLE) that could be used, for instance, to increase selectivity, by isolating the analyte from matrix interfering species, or to enhance selectivity, by concentrating the analyte from a large sample volume. Likewise other sample manipulations, the manual implementation of this mass transfer operation is usually labor-intensive and time-consuming demanding most of the time large amounts of chemicals that could be harmful to the operator, expensive, and environmentally hazardous [1]. Distinct continuous flow strategies have been proposed and exploited to overcome these shortcomings, either by reducing solutions consumption, therefore minimizing waste generation in a more environmental friendly perspective, by limiting operator's intervention and exposure, which minimized the occurrence of errors, by increasing sampling rate, etc.

Since 1978 almost 250 articles exploiting liquid–liquid extraction in flow systems have been published, with a pronounced growth in the number of publications between mid-80s and the early years of the 21st century after which the new publications are scarce (Fig. 1). Flow-based liquid–liquid extraction has been applied to various areas, such as, environmental (38% of the publications), pharmaceutical, clinical and food analysis, among others. The type of detection technique employed in 67% of the publications was UV/visible spectrophotometry, followed by 16% for atomic absorption spectrometry (AAS) and 7% for spectrofluorimetry. As it can be perceived, optical detector systems are favoured mostly because the influence of the organic phase is minimised in such systems. Usually, the selection of a suitable detection system is limited by the presence of trace amounts of one of the immiscible solvents in the other phase.

As it was conceived by the pioneering works of Bergamin et al. [2] and Karlberg et al. [3], a classical liquid–liquid flow injection system is characterized by three main components: a phase segmenter (or confluence), an extraction coil and a phase separator. After the introduction of an aqueous sample, either in a continuous process or in a well-defined volume, into an aqueous stream (which acts as reagent and carrier stream) solutions homogenization occurred and a reaction zone is formed, which is directed towards the segmenter. In the segmenter the two streams of aqueous and organic immiscible phases are put in contact and a single flow of alternate reproducible zones of both phases is generated. Subsequently, in the extraction coil, takes place the mass transfer between the two phases multiple interfaces created by the segmentation process. Finally, in the phase separator, the small aqueous and organic phase segments are continuously split into individual streams, being the one that contains the analyte directed towards the detector for measurement.

In the literature, distinct system manifold configurations of different complexity are described. In the subsequent sections these arrangements and operational modes are discussed in detail. It is important to emphasise that only the works that propose LLE systems with a detection system were considered.

2. Liquid–liquid extraction modalities

2.1. Single extraction

Several publications concerning analytical applications that involved a single extraction operation are listed in Tables 1–5 being the typical system manifold that illustrates its performance, according to the original proposals of Bergamin et al. [2] and Karlberg et al. [3], presented in Fig. 2. Kuban [88] provides a detailed description of various components of the LLE flow system.

As it can be observed in Fig. 3, the manifold comprises a propulsion unit, usually a peristaltic pump, responsible for creating an aqueous stream where the sample is initially introduced. The organic stream can be generated by a peristaltic pump, a liquid chromatographic pump (piston or syringe) [25,75,76], a displacement technique [8,14,89] or a constant gas overpressure (using pressurized inert gas) [43,47,59]. Among the mentioned organic transport units, the displacement techniques are prevalent as they

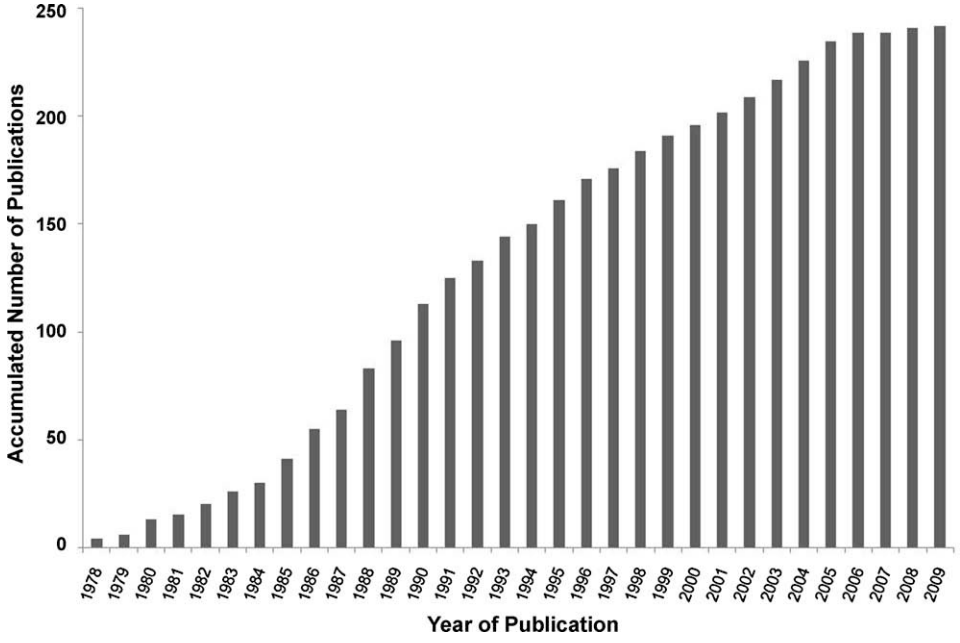

Fig. 1. Annual distribution of publications based on liquid–liquid extraction in flow analysis.

Table 1
Publications that exploit single extraction in flow analysis for water analysis.

Analyte	Matrix	Solvent	Detection	Determination rate (h^{-1})	Linear range	Detection limit	Reference
Aluminum	Drinking, river and wastewater	Chloroform	Fluorimetry	20	20–800 $\mu g\,L^{-1}$	6 $\mu g\,L^{-1}$	[4]
Aluminum	Natural and tap water	Toluene	AAS	30	0.5–200 $\mu g\,L^{-1}$	0.5 $\mu g\,L^{-1}$	[5]
Aluminum	Natural water	Chloroform	Spectr. UV/vis	85	Up to 2 $mg\,L^{-1}$	0.06 $mg\,L^{-1}$	[6]
Anionic surfactants	Industrial water	Chloroform	Spectr. UV/vis	80	0.25–1.25 $mmol\,L^{-1}$	15 $\mu mol\,L^{-1}$	[7]
Anionic surfactants	River and treatment water	Chloroform	Spectr. UV/vis	60	0.04–3.5 $\mu g\,mL^{-1}$	4 $ng\,mL^{-1}$	[8]
Anionic surfactants	River water	1,2-Dichlorobenzene	Spectr. UV/vis	20	Up to 7×10^{-5} $mol\,L^{-1}$	1×10^{-8} $mol\,L^{-1}$	[9]
Anionic surfactants	River water	Chloroform	Spectr. UV/vis	30	Up to 3×10^{-5} $mol\,L^{-1}$	1×10^{-8} $mol\,L^{-1}$	[10]
Anionic surfactants	River water	Toluene, MIBK	Spectr. UV/vis	20	0.1–0.4 $mg\,L^{-1}$	18 $\mu g\,L^{-1}$	[11]
Anionic surfactants	Wastewaters	MIBK	AAS	–	0.1–5.0 $\mu g\,mL^{-1}$	45 $ng\,mL^{-1}$	[12]
Cadmium	Natural water and urine	MIBK	AAS	30	0.006–0.30 $\mu g\,L^{-1}$	2.8 $ng\,L^{-1}$	[13]
Cadmium	Natural water	MIBK	AAS	33	0.06–6.0 $\mu g\,L^{-1}$	0.02 $\mu g\,L^{-1}$	[14]
Calcium	River water	Benzene, chlorobenzene	Spectr. UV/vis	30	Up to 1×10^{-4} $mol\,L^{-1}$	2×10^{-7} $mol\,L^{-1}$	[15]
Copper	Drinking and waste water	MIBK	AAS	40	Up to 900 $ng\,mL^{-1}$	2 $ng\,mL^{-1}$	[16]
Copper	River water	1,2-Dichloroethane	Spectr. UV/vis	22	64.5–4000 $ng\,mL^{-1}$	19.3 $ng\,mL^{-1}$	[17]
Copper	River water	Chloroform	Spectr. UV/vis	64	Up to 2 $mg\,L^{-1}$	0.04 $mg\,L^{-1}$	[18]
Copper	Tap and rain water	Xylene	AAS	80	–	0.02 $\mu g\,mL^{-1}$	[19]
Copper	Water	Chlorobenzene	Spectr. UV/vis	30	Up to 3×10^{-5} $mol\,L^{-1}$	2×10^{-8} $mol\,L^{-1}$	[20]
Dimethoxydithiophosphate	Surface waters	MIBK	AAS	–	25–200 $\mu g\,L^{-1}$	5 $\mu g\,L^{-1}$	[21]
DTAB	Water	MIBK	AAS	35	0.4–9.0 $\mu g\,mL^{-1}$	0.13 $\mu g\,mL^{-1}$	[22]
Fluoride	Water	Hexanol	ICP	36	0.03–1.3 $\mu g\,mL^{-1}$	30 $ng\,mL^{-1}$	[23]
Iron (II)	Natural water	Chloroform	Spectr. UV/vis	85	Up to 2 $mg\,L^{-1}$	0.06 $mg\,L^{-1}$	[6]
Iron (III)	Natural water	Chloroform	Spectr. UV/vis	85	Up to 2 $mg\,L^{-1}$	0.06 $mg\,L^{-1}$	[6]
Lead	Natural water	MIBK	AAS	25	3.0–250.0 $\mu g\,L^{-1}$	1.4 $\mu g\,L^{-1}$	[24]
Oxine-copper	River water	Chloroform	Chemiluminescence	6	28–1100 $\mu g\,L^{-1}$	5.5 $\mu g\,L^{-1}$	[25]
Palladium	Airborne particulate matter	Chloroform	Spectr. UV/vis	20	Up to 12 $mg\,L^{-1}$	0.007 $mg\,L^{-1}$	[26]
Phosphorus	River water	MIBK, Benzene	Spectr. UV/vis	40	Up to 1 $\mu g\,mL^{-1}$	0.1 $ng\,mL^{-1}$	[27]
Potassium	River and tap water	Benzene, chlorobenzene	Spectr. UV/vis	15	Up to 2×10^{-4} $mol\,L^{-1}$	–	[28]
Potassium	River and tap water	Benzene, chlorobenzene	Spectr. UV/vis	12	Up to 8×10^{-5} $mol\,L^{-1}$	–	[29]
Potassium	River water	Benzene	Spectr. UV/vis		Up to 10^{-4} $mol\,L^{-1}$	–	[30]
Potassium	River water	Benzene, chlorobenzene	Spectr. UV/vis	20	Up to 1×10^{-4} $mol\,L^{-1}$	1×10^{-6} $mol\,L^{-1}$	[31]
Potassium	River water	Benzene, chlorobenzene	Spectr. UV/vis	20	5×10^{-6}–1×10^{-4} $mol\,L^{-1}$	–	[32]
Sodium	River and tap water	Benzene, chlorobenzene	Spectr. UV/vis	15	Up to 2×10^{-3} $mol\,L^{-1}$	–	[28]
Sodium	River and tap water	Benzene, chlorobenzene	Spectr. UV/vis	12	Up to 5×10^{-4} $mol\,L^{-1}$	–	[29]
Sodium	River water	Benzene, chlorobenzene	Spectr. UV/vis	20	1×10^{-4}–2×10^{-3} $mol\,L^{-1}$	–	[32]
THAB	Water	MIBK	AAS	35	0.6–14.3 $\mu g\,mL^{-1}$	0.21 $\mu g\,mL^{-1}$	[22]

AAS: atomic absorption spectrometry; DTAB: dodecyltrimethyl ammonium bromide; ICP: inductively coupled plasma; MIBK: methyl isobutyl ketone; Spectr. UV/vis: spectrophotometry UV/visible; THAB: tetraheptyl ammonium bromide.

Table 2
Publications that exploit single extraction in flow analysis for other environmental determinations.

Analyte	Matrix	Solvent	Detection	Determination rate (h^{-1})	Linear range	Detection limit	Reference
Beryllium	Alloys	Carbon tetrachloride	ICP	25	5 $\mu g L^{-1}$ to 100 mg L^{-1}	–	[33]
Cadmium	Petrol	Carbon tetrachloride	Spectr. UV/vis	90	0.8–4.0 mg L^{-1}	–	[34]
Cadmium	Plants, mussel	Carbon tetrachloride	ICP	20	Up to 300 ng mL^{-1}	0.4 ng mL^{-1}	[35]
Copper	Plant digests	Carbon tetrachloride	Spectr. UV/vis	30	50–400 $\mu g L^{-1}$	5 $\mu g L^{-1}$	[36]
Copper	Plants	Chloroform	Spectr. UV/vis	64	Up to 2 mg L^{-1}	0.04 mg L^{-1}	[18]
Copper	Rock	MIBK	AAS	28	10–50 $\mu g L^{-1}$	1 $\mu g L^{-1}$	[37]
Dimethoxydithiophosphate	Agricultural formulation	Chloroform	AAS	–	8.5–17 mg L^{-1}	0.39 mg L^{-1}	[38]
Gold	Coal fly ash, ore	Benzene	Spectr. UV/vis	–	Up to 5 mg L^{-1}	–	[39]
Gold	Rock	MIBK	AAS	28	10–50 $\mu g L^{-1}$	1.8 $\mu g L^{-1}$	[37]
Lead	Pottery glaze	Carbon tetrachloride	Spectr. UV/vis	90	0.2–1.0 mg L^{-1}	–	[34]
Molybdenum	Plant extracts	Isoamyl alcohol	Spectr. UV/vis	30	0.1–1.0 mg L^{-1}	0.05 mg L^{-1}	[2]
Rhenium	Molybdenite, ore	Benzene	Spectr. UV/vis	20	Up to 1.5 mg L^{-1}	–	[40]
Thallium	Coal fly ash, ore	Benzene	Spectr. UV/vis	–	Up to 5 mg L^{-1}	–	[39]
Uranium	Ore leachates	Heptane	Spectr. UV/vis	50	Up to 50 mg L^{-1}	0.1 mg L^{-1}	[41]
Zinc	Soil	Carbon tetrachloride	Spectr. UV/vis	60	Up to 2 mg L^{-1}	–	[42]

AAS: atomic absorption spectrometry; ICP: inductively coupled plasma; MIBK: methyl isobutyl ketone; Spectr. UV/vis: spectrophotometry UV/visible.

Table 3
Publications that exploit single extraction in flow analysis for pharmaceutical determinations.

Analyte	Solvent	Detection	Determination rate (h^{-1})	Linear range	Detection limit	Reference
8-Chloroteophylline	Cyclohexane	Spectr. UV/vis		0.25×10^{-3}–2.50×10^{-3} mol L^{-1}	–	[43]
Amphetamines	MIBK	AAS	30	10–1000 ng mL^{-1}	–	[44]
Amylocaine	1,2-Dichloroethane	AAS	30	3–80 $\mu g mL^{-1}$	2.1 $\mu g mL^{-1}$	[45]
Benzalkonium	1,2-dichloroethane	Spectr. UV/vis	50	5×10^{-7}–2×10^{-7} mol L^{-1}	–	[46]
Benzalkonium chloride	Chloroform	Spectr. UV/vis	40	$1.5–180 \times 10^{-4}$ %(w/v)	–	[47]
Benzethonium	1,2-Dichloroethane	Spectr. UV/vis	30	2×10^{-6}–1×10^{-5} mol L^{-1}	–	[48]
Berberine	1,2-Dichloroethane	Fluorimetry	42	4×10^{-9}–1×10^{-6} mol L^{-1}	8×10^{-10} mol L^{-1}	[49]
Berberine	1,2-Dichloroethane	Spectr. UV/vis	45	1×10^{-6}–1×10^{-5} mol L^{-1}	–	[48]
Bismuth	Dichloromethane	Spectr. UV/vis	20	Up to 20 $\mu g mL^{-1}$	0.24 $\mu g mL^{-1}$	[50]
Bromazepan	MIBK	AAS	–	0.4–4.0 $\mu g mL^{-1}$	0.1 $\mu g mL^{-1}$	[51]
Bromhexine	1,2-Dichloroethane	AAS	30	5–120 $\mu g mL^{-1}$	2.8 $\mu g mL^{-1}$	[45]
Caffeine	Chloroform	Spectr. UV/vis	100	2.74×10^{-4}–8.22×10^{-4} mol L^{-1}	–	[3]
Cetylpyridinium	1,2-Dichloroethane	Spectr. UV/vis	60	5×10^{-7}–2×10^{-7} mol L^{-1}	–	[46]
Codeine	Chloroform	Spectr. UV/vis	60	0.5×10^{-4}–9.0×10^{-4} mol L^{-1}	–	[52]
Diphenhydramine	Cyclohexane	Spectr. UV/vis	120	0.25×10^{-3}–2.00×10^{-3} mol L^{-1}	–	[43]
Enalapril	Dichloromethane	Spectr. UV/vis	80	1.5–60 $\mu g mL^{-1}$	–	[53]
Erythromycin	Chloroform	Fluorimetry	45	1.62–14.7 $\mu g mL^{-1}$	0.68 $\mu g mL^{-1}$	[54]
Imipramine	Chloroform	Spectr. UV/vis	10	3.0–30.0 $\mu g mL^{-1}$	1.0 $\mu g mL^{-1}$	[55]
Lead	Carbon tetrachloride	Spectr. UV/vis	40	Up to 1 mg L^{-1}	0.005 mg L^{-1}	[56]
Lidocaine	Chloroform	Spectr. UV/vis	30	1.4–16.2 $\mu g mL^{-1}$	–	[57]
Neostigmine	1,2-Dichloroethane	Spectr. UV/vis	48	1×10^{-7}–5×10^{-7} mol L^{-1}	–	[58]
Procyclidine hydrochloride	Chloroform	Spectr. UV/vis	15	2.5×10^{-5}–2.0×10^{-4} mol L^{-1}	–	[59]
Thiamin	Chloroform	Fluorimetry	30	3×10^{-4}–6×10^{-4} mg mL^{-1}	–	[60]

AAS: atomic absorption spectrometry; MIBK: methyl isobutyl ketone; Spectr. UV/vis: spectrophotometry UV/visible.

Table 4
Publications that exploit single extraction in flow analysis for clinical determinations.

Analyte	Matrix	Solvent	Detection	Determination rate (h^{-1})	Linear range	Detection limit	Reference
Amphetamines	Urine	MIBK	AAS		10–1000 ng mL^{-1}	–	[44]
Bromazepan	Plasma	MIBK	AAS	–	0.4–4.0 $\mu g mL^{-1}$	0.1 $\mu g mL^{-1}$	[51]
Cadmium	Kidney, liver, pancreas	MIBK	ICP	24	10–2000 ng mL^{-1}	8.7 ng mL^{-1}	[61]
Cadmium	Urine	Chloroform	Spectr. UV/vis	–	Up to 12 $\mu g L^{-1}$	–	[62]
Copper	Liver, pancreas, kidney	Chloroform	ICP	–	Up to 500 ng mL^{-1}	1.5 ng mL^{-1}	[63]
Lithium	Blood serum	Chloroform	Spectr. UV/vis	–	0.4–2 mmol L^{-1}	–	[64]
Lithium	Blood serum	Chloroform	Spectr. UV/vis	100	Up to 2 mmol L^{-1}	1×10^{-7} mol L^{-1}	[65]
Lithium	Blood serum	Dichloromethane	Spectr. UV/vis	–	14–695 mg L^{-1}	–	[66]
Malondialdehyde	Human and rat plasma	MIBK	Spectr. UV/vis	7	Up to 10 μmol L^{-1}	0.27 μmol L^{-1}	[67]
Nickel	Serum, urine, blood, hair	MIBK	AAS	40	Up to 1 $\mu g L^{-1}$	4 ng L^{-1}	[68]
Perchlorate	Serum, Urine	MIBK	AAS	45	0.1–5 $\mu g mL^{-1}$	70 ng mL^{-1}	[69]
Pheniramine maleate	Nasal spray	Chloroform	Spectr. UV/vis	30	0.04–0.23% (w/w)	–	[70]
Phenylphrine hydrochloride	Nasal spray	Chloroform	Spectr. UV/vis	30	0.10–0.57% (w/w)	–	[70]
Zidovudine	Urine	Methylene chloride	Spectr. UV/vis	–	0.9×10^{-4}–4.0×10^{-4} mol L^{-1}	1.2×10^{-8} mol L^{-1}	[71]

AAS: atomic absorption spectrometry; ICP: inductively coupled plasma; MIBK: methyl isobutyl ketone; Spectr. UV/vis: spectrophotometry UV/visible.

Table 5
Publications that exploit single extraction in flow analysis for food and chemistry analysis.

Analyte	Matrix	Solvent	Detection	Determination rate (h^{-1})	Linear range	Detection limit	Reference
Aminocarb	Cow's milk	Hexachlorocyclohexane	GC	–	50–800 ng mL^{-1}	20 ng mL^{-1}	[72]
Arsenic	Beer, tomatoes, mussels	Xylene	ICP	–	Up to 100 μg mL^{-1}	2 ng mL^{-1}	[73]
Benthiocarb	Cow's milk	Hexachlorocyclohexane	GC	–	35–700 ng mL^{-1}	14 ng mL^{-1}	[72]
Bitterness compounds	Beer	Iso-octane	Spectr. UV/vis	60	10–50 BU		[74]
Caffeine	Beverages	Chloroform	Spectr. UV/vis	60	Up to 70 mg L^{-1}	–	[74]
Caffeine	Beverages	Methylene chloride	Spectr. UV/vis	–	0.9×10^{-4}–4.0×10^{-4} mol L^{-1}	2×10^{-9} mol L^{-1}	[71]
Carbaryl	Cow's milk	Hexachlorocyclohexane	GC	–	25–500 ng mL^{-1}	10 ng mL^{-1}	[72]
Carbofuran	Cow's milk	Hexachlorocyclohexane	GC	–	50–1000 ng mL^{-1}	20 ng mL^{-1}	[72]
Cationic surfactants	Industrial products	Chloroform	Spectr. UV/vis	60	0.3–3.0 mmol L^{-1}	–	[75]
Chromium (VI)	Reference material	MIBK	AAS	10	0.01–0.80 μg L^{-1}	3.2 μg L^{-1}	[76]
Cobalt	Tool steels	Chloroform	Spectr. UV/vis	20	Up to 20 μg mL^{-1}	0.23 μg mL^{-1}	[77]
Dichromate	Steels	Chloroform	Spectr. UV/vis	24	Up to 20 μg mL^{-1}	0.44 μg mL^{-1}	[78]
Ethanol	Beverages	Chloroform	FTIR	2	Up to 15.0% (v/v)	0.03% (v/v)	[79]
Free fatty acid	Vegetable oil	Toluene	Spectr. UV/vis	130	4×10^{-5}–6×10^{-3} N	–	[80]
Iodide	Table salt	Xylene	ICP	20	100 ng mL^{-1} to 100 μg mL^{-1}	20 ng mL^{-1}	[81]
Methiocarb	Cow's milk	Hexachlorocyclohexane	GC	–	5–100 ng mL^{-1}	2 ng mL^{-1}	[72]
Nitrate	Meat, Vegetables	MIBK	AAS	20	0.2–2.2 μg mL^{-1}	–	[82]
Nonionic surfactants	Detergents	1,2-Dichloroethane	Spectr. UV/vis	–	0.02–1.2 mg L^{-1}	–	[83]
Perchlorate	Reagents	Benzene	Spectr. UV/vis	20	Up to 2.5 μg mL^{-1}	0.036 μg mL^{-1}	[84]
Perchlorate	Reagents	MIBK	Spectr. UV/vis	30	0.008–1.00 μg mL^{-1}	0.003 μg mL^{-1}	[85]
Permanganate	Steels	Chloroform	Spectr. UV/vis	24	Up to 25 μg mL^{-1}	0.58 μg mL^{-1}	[86]
Propoxur	Cow's milk	Hexachlorocyclohexane	GC	–	50–800 ng mL^{-1}	20 ng mL^{-1}	[72]
Total aliphatic amines	Wine, beer	Chloroform	Spectr. UV/vis	30	1.3–132 mg L^{-1}	0.5 mg L^{-1}	[87]

AAS: atomic absorption spectrometry; FTIR: fourier transform infrared spectroscopy; GC: gas chromatography; ICP: inductively coupled plasma; MIBK: methyl isobutyl ketone; Spectr. UV/vis: spectrophotometry UV/visible.

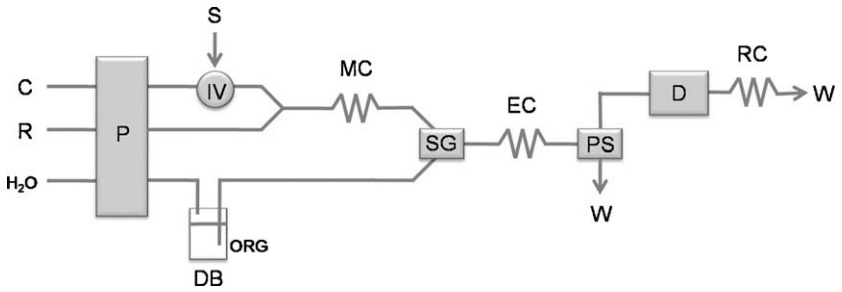

Fig. 2. Typical liquid–liquid extraction flow analysis system manifold. C: carrier; R: reagent; P: propulsion unit; S: sample; IV: injection valve; MC: mixing coil; DB: displacement bottle; ORG: organic solvent; SG: segmenter; EC: extraction coil; PS: phase separator; D: detector; RC: restriction coil; W: waste.

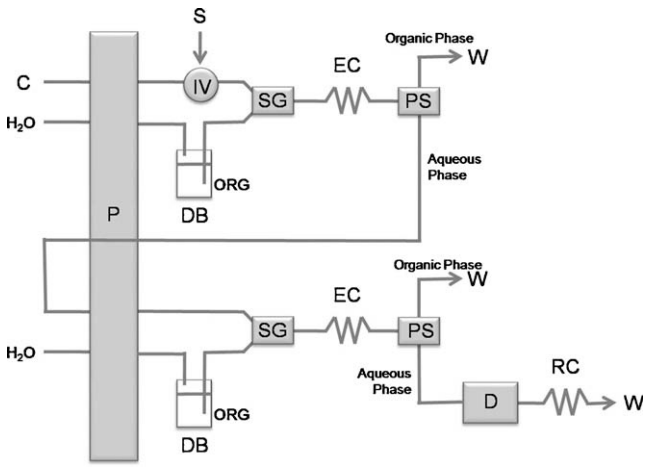

Fig. 3. System manifold representing a two-stage extraction (multiple extractions). C: carrier; P: propulsion unit; S: sample; IV: injection valve; DB: displacement bottle; ORG: organic solvent (with or without extractant); SG: segmenter; EC: extraction coil; PS: phase separator; D: detector; RC: restriction coil; W: waste.

offer the easiest, most economic and most straightforward alternative. In a displacement bottle, for instance, an aqueous stream is propelled by means of a peristaltic pump into a closed container that is completely filled with an organic solvent, which is therefore dislocated at a constant flow rate and forced into the flow system. This way, the need for expensive liquid chromatographic pumps as well as for solvent-resistant tubing along with the presence of unwanted stream pulses or irregularities of the flow rate (either by using peristaltic pumps or a constant gas overpressure) is circumvented.

One of the three principal pieces of the LLE flow system, the segmenter, is responsible for phase segmentation. Segmenters can be divided in two different groups: continuous flow segmenters (including non-coaxial segmenters like T-, Y- or W-pieces and coaxial segmenters) and mechanical segmenters (such as pneumatic or motor-driven loop injector and magnetic valves).

The segmentation system is commonly positioned after the injection valve. However, in some instances the flow manifold exhibits the segmentation system prior to the injection valve [90]. In this case, the dispersion level attained is minimised since samples are directly injected into the segmented rather than into the unsegmented flow stream. In effect, the dispersion process that occurs during the transport of the sample through the mixing/reaction

coil before entering the segmenter and the phase separator is eliminated.

The extraction coil is the manifold component where occurs the transfer of the analyte between phases, a process that is affected by factors such as the residence time and the liquid–liquid interface surface area as well as the hydrophilic or lipophilic character of coil tubing material (depending on whether the sample will be extracted from aqueous into the organic phase or vice versa) and the coil dimensioning and configuration.

The phase separator is probably the most significant component of a liquid–liquid extraction flow system. In fact, the phase (acceptor) that will participate in the succeeding process leading to the measurement step should be kept completely free from any contamination from the feed phase in order to assure, at detection, an analytical signal with an adequate and reproducible profile avoiding the occurrence of fluctuations that could affect both precision and accuracy. Therefore, the phase separator is designed with a configuration that could handle small volume segments of the two immiscible phases thus preventing detrimental/dispersion phenomena which, in addition to a decrease in sensitivity, result in a loss of reproducibility.

There are several types of phase separators that resort to distinct separation modes: density (chamber) separators that rely on gravity to separate the phases; affinity separators, which are made both of lipophilic and a hydrophilic materials being the separation process based on the affinity difference between the phases and the separator chamber materials; membrane phase separators that are based on selective permeability of a microporous membrane towards the phase which wets the membrane material. The membrane phase separator present some advantages regarding other types of separators like the possibility of utilisation with a large variety of water immiscible solvents since a density/affinity difference between the two solvents is not required; it exhibits a smaller internal volume that reduces the dispersion or dilution of the analyte; it is compatible with high flow rates, which can reduce the time of the analysis. In practice, membrane phase separators are superior to affinity/density phase separators, but they have the disadvantage of requiring a frequent membrane replacement and for having a poor recovery percentage of the phase that contains the analyte.

By applying two phase separators in series with a single detector some analytical approaches allow the simultaneous monitoring of both organic and aqueous phases [7,23,67,70,71]: one of the phase separators is accountable for isolating the organic phase which is directed towards the detector flow-cell, while the second one is responsible for accommodating the aqueous phase with minor traces of the organic phase. The separated aqueous stream flows through the second detector flow-cell to measure water-soluble species. In another proposal, the manifold incorporated two parallel detectors [91] instead of one detector with two flow-through cells, for the simultaneous determination of extractable and non-extractable species. A similar performance was achieved using only one phase separator (with a dual membrane) and two detectors [43]. When the detector is equipped with a temperature-controlled cell holder, the separated phase can be measured based in the thermochromism of the ion-associated complexes formed in the reaction [46,48,92].

An important aspect to be taken into consideration when using a membrane separator is the utilisation of restriction coils or needle valves after the detector. They could be used for regulating the overpressure across the phase separator membrane, and can be arranged to produce a backpressure that forces the organic phase through the hydrophobic membrane. In addition, these components can be used to remove the remaining traces of the organic solvent, together with the aqueous phase, into waste.

Coupling a LLE system with an AAS detector involves a more sophisticated manifold configuration and operation [93]. The analytical system comprises two autonomous sections, since different flow rates are required for the extraction and the nebulisation stages, and relies on a continuous sample introduction. After the separation of the organic phase in the phase separator, a fraction of it is periodically injected (by means of an injector sampling loop) into an independent carrier stream which is continuously pumped into the spectrometer [12,14,16,22,44,45,51,69,76,82,89,94–97]. This system enables the extraction system to work independently from the nebulizer feed system. Moreover, the whole system is environmental friendly since by working in a closed manifold, it prevents the evaporation of the involved organic solvents avoiding atmospheric contamination.

Implementation of multi-commutation for performing LLE was also achieved [98–100]. The flow network comprised a set of solenoid valves and provided a significant reduction of reagent consumption. Moreover, the extraction was based in the single extraction mode, although a segmenter was not needed since the solenoid valves were capable of carrying out the segmentation process. Nevertheless, some drawbacks, such as an increase in the washing time caused by organic solvent adsorption and a limitation of the pre-concentration factor, were noticed.

2.2. Multiple extractions

In a multiple extractions operational mode the separation process is repeated several times by using either the same or a different extractant in the successive stages [101–103]. Its applicability is associated with the isolation of the analyte from complicated sample matrices. The system manifold is similar to the one used in single extraction, however it includes more components that are directly related to the number of extractions performed (Fig. 3).

2.2.1. Closed-loop systems

In this type of system, a continuous extraction of the analyte into a small volume of the organic phase circulating in a closed loop is achieved [104,105]. The sample is continuously introduced via a four-way switching valve, being directed to a four-way segmenter where it is interspaced with the organic phase. The segmented stream is directed to an extraction coil followed by a phase separator. Here, the aqueous fraction is removed to waste while the organic phase is pumped back to the segmenter where it is put in contact with a new incoming sample aliquot for further enrichment. As the organic phase is repeatedly sent back into the closed loop system (thus enabling a longer residence time) higher enrichment factors are achieved. It is important to notice the existence of a "make-up" or "refill" flow of the organic solvent to compensate the volume losses due to the formation of small bubbles of organic vapours, formation of small droplets of aqueous phase, and solubility of the organic solvent in the aqueous phase [104]. A schematic manifold is pictured in Fig. 4.

This approach allows the automated pre-concentration and purification of analytes into a fixed volume of organic phase circulating in a closed loop, which means that the system is able to strip the analyte and/or remove interferences without the need for extra phase separators. However, the system presents a disadvantage since it only can be used when a relatively large sample volume is available.

2.3. Back extraction

This arrangement offers a multi-stage extraction in which the aqueous sample is initially extracted into an organic medium and then back-extracted into another aqueous phase, where the measurements are carried out (Fig. 5) [106–108].

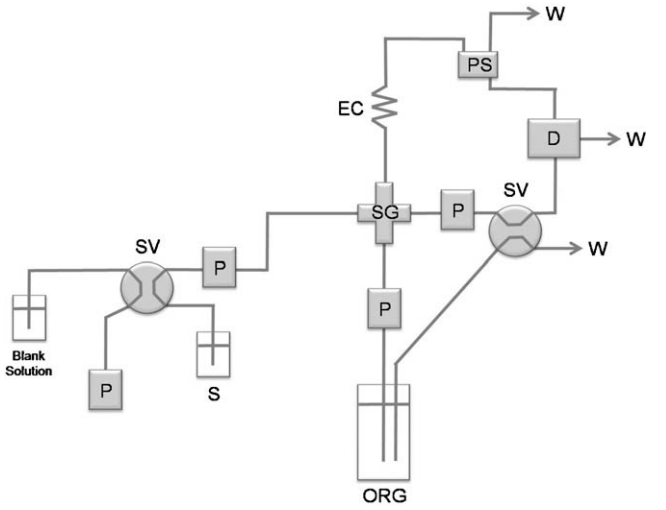

Fig. 4. Closed-loop system manifold. P: propulsion unit; S: sample; SV: switch valve; ORG: organic solvent; SG: segmenter; EC: extraction coil; PS: phase separator; D: detector; W: waste.

It is mostly applied to the determination of trace metals by electrothermal AAS (ETAAS) since the final extract has the advantage of being an aqueous solution. The introduction of organic eluates into flame AAS is beneficial because it facilitates the creation of an increased number of smaller droplets that eventually pass through the nebulizer and into the flame, thus improving sample insertion (in addition these could act as fuel or flame promoters). However, its use for ETAAS should be avoided because organic solvents, due to a surface tension lower than that of water, generally have a tendency to distribute alongside the length of the graphite tube. When it comes to inductively coupled plasma (ICP), organics are disadvantageous because they might give rise to the generation of interfering ions. Therefore, if an aqueous sample is to be pretreated by extraction and the analyte determined by either ETAAS or ICP, it is preferable, or actually almost compelling, to do the extraction–back extraction, where the analyte is first extracted into an organic solvent and then back extracted into an aqueous solution, which is then introduced in the detector [109].

In fact, after the first phase separation, the partitioned organic phase containing the metal chelates is directed towards a second segmenter where it is intercalated with an aqueous phase containing a striping reagent. This dislocates the metal from the chelates formed in the organic phase by promoting the formation of new chelates with stronger interactions. The process of back extraction into the aqueous phase takes subsequently place in the second extraction coil and after a second phase separator the organic phase is discarded to waste while the aqueous phase is sent to the detector for measurement. Actually, the second extraction does not contribute significantly to the sample enrichment but greatly simplifies the ETAAS and ICP measurements.

2.4. Systems without phase separation

One of the most straightforward LLE strategies is the single-line manifold without phase separation, which provides an enhanced sensitive due to the absence of phase separator and thus the consequent suppression of the additional sample dispersion associated with its use. In this type of approach, the sample is typically injected into a continuous organic phase stream (that usually contains a colour developing reagent) and then flows through the extraction coil, where occurs the formation of an extractable complex between the analyte and the dissolved reagent that is measured in the detector (Fig. 6A) [110]. Alternatively, a defined organic phase volume can be injected into a continuous sample flowing stream (the loop of the injection valve is usually fed by means of a displacement bottle), which is then directed towards the detection system (Fig. 6B) [4,111]. In these two examples, not only the phase separator is needless but they do not also require a segmenter (replaced by an injection valve). Nevertheless, some applications still resort to the segmenter (Fig. 6C) [4,111–115]. When a single segment of organic solvent (Fig. 6B) is injected into an aqueous stream higher pre-concentration ratios and sampling frequencies are achieved but the analytical application range is more limited and the precision is inferior regarding the reversed situation (Fig. 6A). The reduced repeatability seemed to be caused by a partial retention of the organic phase at the flow-cell by adsorption onto the walls of the chamber. Although a larger consumption of sample solution is observed in mode B, due to its continuous insertion, a drastic saving of organic phase is attained.

The most suitable detection systems used with this extraction strategy are generally AAS, fluorimetry and scanning spectrophotometry (sometimes resorting to a capillary flow cell). In this latter case, the detection system is placed perpendicularly to the flow direction and has the advantage of allowing the continuous sequential monitoring of the small aqueous and organic phases segments [112,113,116]. These have well-defined volumes and are generated through the use of, for instance, alternately operated stepper-motor driven syringe pumps or by using appropriate segmenters. The resulting uniform segmentation makes possible a subsequent digital phase separation under computer control, the analytical signals being measured and processed mathematically. A diode array spectrophotometer can also be used to monitor the segmented flow [114,115] with a multi-task performance: a non-specific wavelength

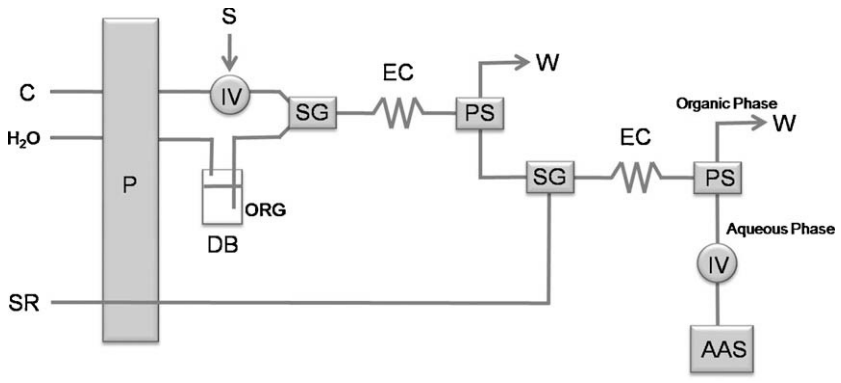

Fig. 5. System manifold illustrating back extraction in flow analysis. C: carrier; SR: stripping reagent; P: propulsion unit; S: sample; IV: injection valve; DB: displacement bottle; ORG: organic solvent (with or without extractant); SG: segmenter; EC: extraction coil; PS: phase separator; AAS: atomic absorption spectrophotometer; W: waste.

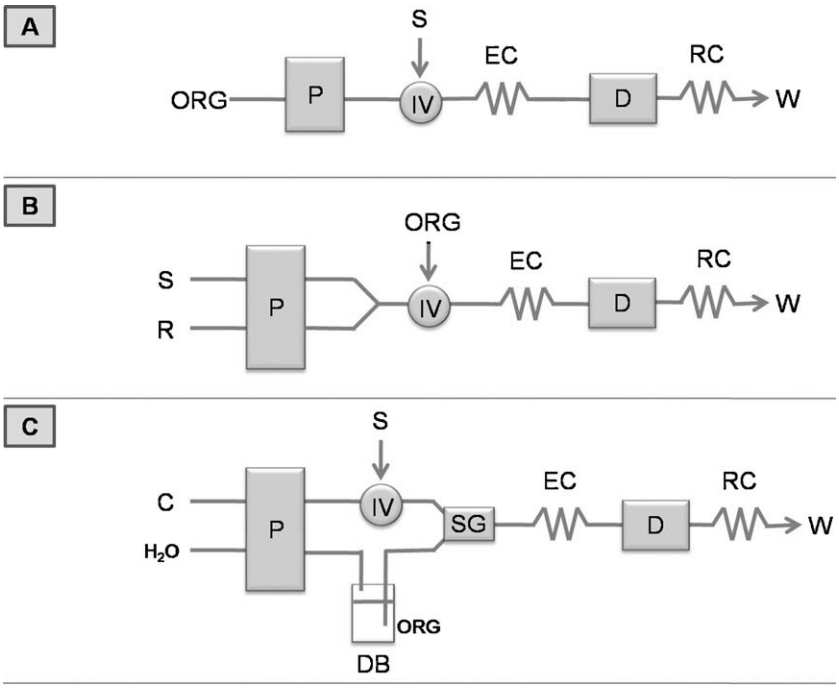

Fig. 6. Types of system manifold without phase separation. C: carrier; R: reagent; P: propulsion unit; S: sample; IV: injection valve; DB: displacement bottle; ORG: organic solvent (with or without extractant); SG: segmenter; EC: extraction coil; D: detector; RC: restriction coil; W: waste.

is used to recognize the phases and a second one monitors the analyte concentration. A third wavelength could be also used to carry out the correction of signal fluctuations.

2.4.1. Zone sampling mode

One important feature of zone sampling approach is the ability to isolate individual segments of organic or aqueous phases without a phase separation unit [117]. In a first stage, a segment of extractant (organic or aqueous phase) is injected into a flowing sample stream. If required, this sample stream could have been previously merged with reagent streams. Subsequently, the extractant segment is isolated as it flows through the loop of an electronically actuated injection valve. The actuation of this valve is determined by sensing its loop-filling status with conductivity probes (inserted in the load and waste ports) or by appropriate time-based control. When the loop of the valve is completely full of the desired phase, this is switched to the injection position in order to enable the entire content of the loop to be injected into a miscible carrier stream flowing towards the detector. This method provides the advantage of allowing the carrying out of further chemical treatments of the analyte, prior to its detection.

2.5. Systems without phase segmentation and separation

In this LLE strategy the analytical manifold does not include a phase segmenter and a phase separator.

2.5.1. Systems with a semi-permeable membrane or sorptive column

In this type of systems, neither segmentation nor separation of the phases are required since aqueous and organic streams are individually directed into both sides of a membrane unit [118–123]. The extracted analyte is transferred from the aqueous stream across the semi-permeable membrane (which is saturated with the organic solvent) into the organic phase or vice versa, being then pumped into the detector's flow cell. Although this system solves inherent problems associated with the presence of a segmenter and a phase separator, it exhibits lower extraction efficiency owing to the restricted surface area and short contact time between the two immiscible phases.

An alternative to the membrane unit that provided higher extraction efficiency consisted of the use of a column filled with a resin that selectively adsorbs the aqueous phase [124]. In this case, the aqueous sample is injected into an organic stream and extraction of the analyte occurs during its transport towards and through the column. The aqueous phase is selectively absorbed and only the organic phase flows into the detector. The principal drawback of this approach is that a higher backpressure is sometimes observed at the column.

2.5.2. Systems with supported liquid membrane

A liquid membrane is formed by a thin layer of organic phase (usually with dissolved reagents) between two aqueous phases of different composition. This thin layer of organic phase can be immobilized onto a suitable inert microporous support, which when interposed between two appropriate aqueous solutions is termed supported liquid membrane (SLM).

In this three-phase extraction technique [1,93,120–122, 125–134], analytes are extracted from a continuously flowing aqueous sample through an organic liquid phase into another usually temporally stagnant, aqueous phase. This results in the selective transport of the analyte, from the feed (donor) solution through the SLM to a stripping (acceptor) solution. The organic phase is held typically in a microporous, hydrophobic membrane by capillary forces.

SLM extraction technique can be combined with immunological recognition [131,133], by introducing analyte-specific antibodies as trapping reagents in the SLM acceptor. The pH of the donor phase is adjusted to render the analyte uncharged increasing its lipophilic character and thus its extractability into the organic membrane. The uncharged analyte (antigen) is extracted from the donor to the acceptor as a result of the established concentration gradient, which is upheld by the binding of the antigen to the analyte-specific antibody, in the acceptor. The analyte is thus trapped in the acceptor

as analyte–antibody complexes that are themselves excluded from the SLM due to their low solubility in organic media [133].

Accordingly, using SLMs presents several benefits, such as the use of small amounts of organic reagents, minimum losses of organic reagents, reduced handling of potentially toxic reagents, simplicity and ease of automation. Since the use of organic solvents in large amounts is unnecessary, the analysis is more economical and environmental friendly, and in some cases, less hazardous. However, the limited lifetime of the liquid membrane, especially when polar organic solvents are used, its susceptibility to deterioration by some types of sample matrices as well as the relatively low enrichment rate that results in long enrichment times when very large enrichment factors are needed, constitute the main drawbacks.

To overcome this limitations another alternative for the use of SLM was proposed: firstly the analyte was transferred into the organic phase; then the organic phase was transported to the SLM equipment where an organic film was formed on the surface of the membrane; finally the analyte passes through the liquid membrane, and was trapped by the acceptor [135,136]. In this case, the liquid membrane is continuously renewed which markedly enhanced its stability.

2.5.3. Iterative reversal systems

This operational mode is based on simplified configurations, with no segmenter or separation units. However, the methodology is quite different since it relies on the reversal of the flow direction for a pre-selected number of cycles [137–142].

Its unique feature is the positioning of the detector in the loop of an injection valve, which is filled with the organic phase (Fig. 7). This single plug of organic phase is inserted into the aqueous phase, which contains the analyte, by switching the injection valve to the emptying position creating two liquid–liquid interfaces. The flow is subjected to an iterative reversal by means of a programmable electronic unit that controls the propulsion unit and the gradual enrichment of the organic phase with the analyte is continuously monitored. The flowing stream is propelled in one direction and when the interfaces are close to the detector flow cell the flow is reversed. As a consequence, some detection interfering factors are kept away from the flow cell, avoiding the occurrence of fluctuations in the analytical signal resulting from changes in the refractive indexes or in viscosity differences between the two phases.

Although essentially similar to the one described above, in some works the detector was not placed in the loop of the injection valve, being instead placed after [139,140] or before [141,142] the injection valve. In the later case, ultrasounds were used to accelerate the extraction process. However, the inherent heterogeneity of the ultrasonic irradiation in the water bath containing the extraction coil was an important parameter to be taken into consideration. This operation mode allows continuous monitoring of the analytical signal with a single detector in a multi-detection approach that provides additional useful information from a single sample insertion, which could be used for distinct analytical purposes. For instance, it is possible to continuously study the analyte transfer between both phases and its dispersion in the organic phase, as well as to implement conventional reaction rate measurements for theoretical and practical purposes. The only limitation is the need for an electronic component to obtain programmable and reproducible cycles.

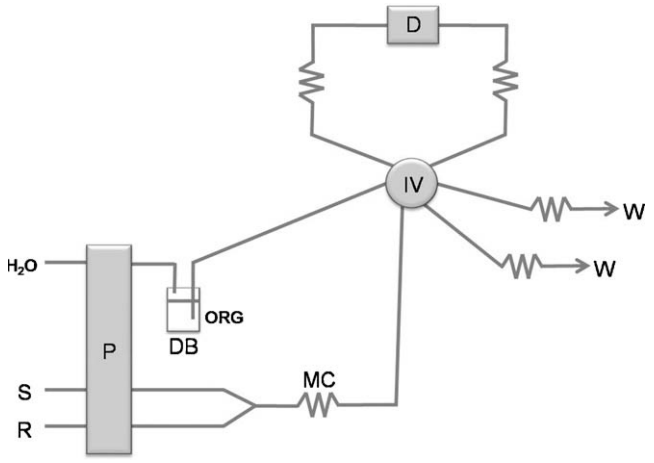

Fig. 7. Iterative reversal system manifold. P: propulsion unit; S: sample; R: reagent; MC: mixing coil; DB: displacement bottle; ORG: organic solvent; IV: injection valve; D: detector; W: waste.

2.5.4. Wetting-film mode

The wetting-film mode is exploited in systems without segmenter and phase separator [1,93,143,144]. Typically, the organic solvent is introduced into the extraction coil to coat the inner wall of the Teflon tubing, forming a pseudo-stationary phase. Next, air (or in some cases water) is introduced to stabilize the formed wetting-film and to prevent the direct contact of the organic coating solvent and the aqueous sample solution. In this process, the solvent flow is impeded due to hydrophobic interactions with the walls of the tubing, leaving the extractant phase adhered to the reactor [143]. After this, the sample is introduced into the extraction coil and the desired analyte is extracted into the organic wetting-film. In a last stage, an elution solution is inserted through the extraction coil to back-extract (elute) the analyte from the wetting-film and to carry it out towards detection for proper measurement. The elution could be also achieved by washing out of the analyte containing film. Before starting a new cycle the extraction coil is rinsed; actually, the extracting film is aspirated and removed during each analysis cycle so that a newly prepared and fresh organic phase is always available, which makes regeneration and re-use not necessary. Furthermore, this technique is characterized by a low consumption of organic solvent and, if necessary, it can allow the quantification of both extracted and unextracted components from a single sample insertion. The most critical aspects of this approach are the thickness and stability of the wetting film, as they are related to reproducibility, sensitivity and extraction capacity.

Various types of flow systems have resorted successfully to the wetting-film mode (Table 6), namely, flow injection [147,149,152,160], sequential injection [145,148,150,151,153–155] and multi-syringe [146]. Saving of reagents/sample and lowering of waste production over flow injection is achieved either by sequential injection or by multi-syringe, given that the required volumes are propelled into the manifold only at the moment of the determination. Moreover, in multi-syringe, as reagents and sample need not be loaded in the holding coil of a selection valve, sampling frequencies showed a significant increase with respect to sequential injection.

2.5.5. Utilisation of chromatomembranes

An interesting alternative to the typical flow extraction procedures arises from the application of chromatomembrane cells (CMC) in flow systems (Table 6) [144,156–159,161]. The CMC consists of a rectangular block of PTFE containing two types of pores (micropores and macropores) as its main component. Polar liquids fill the macropores whereas the micropores remain available only for non-polar liquids or gases. The macropores are selected in such a way as to assure that their capillary pressure is negligibly small and does not hinder the transport of the polar liquid phase. In contrast, the micropores are so narrow that the capillary pressure prevents the polar liquid phase from penetrating them. At the same time the micropores must assure a substantial permeability

Table 6
Publications involving systems that rely on wetting-film and chromatomembrane techniques.

Operational mode	Analyte	Matrix	Solvent	Detection	Determination rate (h^{-1})	Linear Range	Detection limit	Reference
Wetting-film	2-Nitrophenol	Distilled, tap, ground, sea and waste water	1-Octanol	Spectr. UV/vis	4	3.0–32.8 μmol L^{-1}	0.26 μmol L^{-1}	[145]
	2-Nitrophenol	Distilled, tap, ground, sea and waste water	1-Octanol	Spectr. UV/vis	11	2–43 μmol L^{-1}	0.11 μmol L^{-1}	[146]
	3-Nitrophenol	Distilled, tap, ground, sea and waste water	1-Octanol	Spectr. UV/vis	4	4.3–80.9 μmol L^{-1}	0.33 μmol L^{-1}	[145]
	3-Nitrophenol	Distilled, tap, ground, sea and waste water	1-Octanol	Spectr. UV/vis	11	4–110 μmol L^{-1}	0.46 μmol L^{-1}	[146]
	4-Nitrophenol	Distilled, tap, ground, sea and waste water	1-Octanol	Spectr. UV/vis	4	0.4–6.8 μmol L^{-1}	0.035 μmol L^{-1}	[145]
	4-Nitrophenol	Distilled, tap, ground, sea and waste water	1-Octanol	Spectr. UV/vis	11	0.4–10 μmol L^{-1}	0.07 μmol L^{-1}	[146]
	8-Chlorotheophylline	Motion sickness tablets	Hexanol	Spectr. UV/vis	12	2–6 mmol L^{-1}	–	[147]
	Bromothymol blue	Synthetic	Benzene	Spectr. UV/vis	30	Up to 2 mg L^{-1}	50 μg L^{-1}	[148]
	Cadmium	Tap, river, sea and waste water	Diisobutyl ketone	AAS	22	1.5–45 μg L^{-1}	0.7 μg L^{-1}	[149]
	Cadmium	Tap water, soil and urine	Ethanol	Spectr. UV/vis	27	1–20 mg L^{-1}	–	[150]
	Chromium (VI)	Tap, lake and sea water	Octanol, MIBK	Spectr. UV/vis	17	Up to 100 μg L^{-1}	2.0 μg L^{-1}	[151]
	Cobalt	Tap water, soil and urine	Ethanol	Spectr. UV/vis	27	1–20 mg L^{-1}	–	[150]
	Copper	Tap and river water	MIBK	AAS	30	Up to 100 μg L^{-1}	0.2 μg L^{-1}	[152]
	Copper	Tap water, soil and urine	Ethanol	Spectr. UV/vis	27	1–20 mg L^{-1}	–	[150]
	Diphenhydramine	Motion sickness tablets	Hexanol	Spectr. UV/vis	12	2–6 mmol L^{-1}	–	[147]
	Iron (III)	Tap water, soil and urine	Ethanol	Spectr. UV/vis	27	1–10 mg L^{-1}	–	[150]
	Lead	Tap water, soil and urine	Ethanol	Spectr. UV/vis	27	1–20 mg L^{-1}	–	[150]
	Mercury	Tap water, soil and urine	Ethanol	Spectr. UV/vis	27	1–10 mg L^{-1}	–	[150]
	Molibdenium	Aqueous	Toluene, THAB	Spectr. UV/vi	25	5–80 ng mL^{-1}	2.4 ng mL^{-1}	[153]
	Strontium 90 isotope	Mineral, ground and seawater, soil and powdered milk	1-Octanol	β-Counter	< 3	0.07–0.30 Bq	–	[154]
	Vanadium	Aqueous	Benzene	Spectr. UV/vis	15	0.05–0.3 μg mL^{-1}	12 ng mL^{-1}	[155]
	Zinc	Tap water, soil and urine	Ethanol	Spectr. UV/vis	27	1–20 mg L^{-1}	–	[150]
Chromatomembrane	Anionic surfactants	Aqueous	Chloroform	Spectr. UV/vis	–	0.02–5.0 mg L^{-1}	–	[156]
	Copper	Pharmaceuticals	Chloroform	Spectr. UV/vis	10	0.05–0.8 μg mL^{-1}	0.04 μg mL^{-1}	[157]
	Oil products	Natural water	Hexane	Fluorimetry	6	1–1000 μg L^{-1}	–	[158]
	Phenol	Natural and waste waters	Chloroform, hexane	Spectr. UV/vis	15	1.0–10 μg L^{-1}	–	[159]
	Phenol	Natural water	Tributylphosphate	Fluorimetry	6	0.5–100 μg L^{-1}	–	[158]
	Zinc	Pharmaceuticals	Chloroform	Spectr. UV/vis	10	0.05–0.6 μg mL^{-1}	0.04 μg mL^{-1}	[157]

AAS: atomic absorption spectrometry; Bq: becquerel; MIBK: methyl isobutyl ketone; Spectr. UV/vis: spectrophotometry UV/visible; THAB: tetraheptylammonium bromide.

of the biporous medium for the gas or non-polar liquid flow. The supply of the two phases into the CMC and their final separation occurs by suitable placing of microporous PTFE membranes that guarantees the flow-through of both phases. Consequently, the two phase streams move independently due to the presence of these two types of significantly differing pores size in the PTFE [157,162].

The analytical procedure involved in a flow system that comprises a CMC consists basically in filling the micropores with organic phase; in delivering the aqueous phase containing the analyte to the CMC; extraction of the analyte into the organic phase in the micropores; elution of the extracted analyte with an organic phase and transport towards the detector for measurement. The CMC is responsible for either the pre-concentration of the analyte and for the separation of the phases. This way, the flow system does not need a segmentation nor a separation unit, making the use of a CMC a very promising technique because the three steps of the extraction procedure are combined and carried out in just one small device. Moreover, it demands only very small volumes of organic phase and, if required, it can enable continuous extraction or pre-concentration. The main disadvantage of this method is the clogging of the biporous hydrophobic matrix by suspended particles present in the samples, which could demand frequent replacement of the chromatomembrane cells.

2.5.6. Optosensing systems

Another approach that avoids the use of the segmenter, and also the extraction coil and phase separator, relies in the utilisation of a small plug of organic phase that is introduced in a conventional cuvette placed in the detector's cuvette-holder. The aqueous phase containing the analyte is passed through the organic phase and its gradual enrichment with the analyte is continuously monitored. This way, a large volume of sample can be passed through a small organic volume to achieve a high pre-concentration factor that depended on the overall sample volume [163–165].

2.6. New trends

As in many other fields, miniaturization of LLE procedures has gathered a deep interest and has been the focus of many recent deployments. The miniaturization of solvent extraction methodologies leads to a significant reduction in solutions consumption (solvent and reagent) and an inherent decrease in liquid wastes generation [93]. Among the various techniques exhibiting a high analytical potential single-drop microextraction (SDME) and dispersive liquid–liquid microextraction (DLLME) are being the most exploited. SDME was initially based on the utilisation of a small droplet of organic solvent, formed and suspended at the tip of a micro-syringe needle, which was immersed into the aqueous sample [166–169]. The continuous supply of fresh aqueous sample by means of a continuous flow system led to a marked improvement in terms of extraction efficiency [170–174]. DLLME is based on ternary components systems relying on a combination of extraction and disperser solvents with high miscibility in both aqueous and extractant. The solvent mixture is injected into the aqueous sample flowing stream resulting in the formation of fine droplets of extraction solvent dispersed in the aqueous phase. The droplets containing the extracted analyte are subsequently retained in a microcolumn being later eluted towards detection [175].

3. Conclusions

After 30 years of the pioneering works of Bergamin and Karlberg, several different strategies have been developed for carrying out liquid–liquid extraction in flow analysis. The improvements were significant, as the authors tried to simplify the original proposals solving some of its inherent drawbacks. Most of these advances were innovative as they offer alternative means to simplify the manifolds configuration either by avoiding the use of the segmenter or of the phase separator.

Liquid–liquid extraction in flow analysis, when compared with manual methods, contributed not only for the reduction of the consumption of organic solvents, reagents and samples, leading to less expensive analysis, but assured more environmental friendly analytical solutions with a decrease in waste generation and in the operators' exposure risks. Moreover, working as a closed system, contamination is avoided, and the combination of high determination rate, low maintenance level and easy handling makes it one of the most interesting analytical operations to be implemented in analytical flow systems, being able to be applied in a variety of areas, most of them requiring the separation and pre-concentration of analytes from very complex matrices.

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Exploitation of a single interface flow system for on-line aqueous biphasic extraction☆

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ABSTRACT

The exploitation of aqueous biphasic extraction is proposed for the first time in flow analysis. This extraction strategy stands out for being environmentally attractive since it is based in the utilization of two immiscible phases that are intrinsically aqueous. The organic solvents of the traditional liquid–liquid extractions are no longer used, being replaced by non-toxic, non-flammable and non-volatile ones. A single interface flow analysis (SIFA) system was implemented to carry out the extraction process due to its favourable operational characteristics that include the high automation level and simplicity of operation, the establishment of a dynamic interface where the mass transfer occurred between the two immiscible aqueous phases, and the versatile control over the extraction process namely the extraction time. The application selected to demonstrate the feasibility of SIFA to perform this aqueous biphasic extraction was the pre-concentration of lead. After extraction, lead reacted with 8-hydroxyquinoline-5-sulfonic acid and the resulting product was determined by a fluorimetric detector included in the flow manifold. Therefore, the SIFA single interface was used both as extraction (enrichment) and reaction interface.

1. Introduction

Conventional liquid–liquid extraction involves the utilization of an organic solvent and an aqueous solution as the two immiscible phases. Some of its characteristics, such as the possibility to use high number of different solvents, extractants and aqueous phases, make solvent extraction an attractive and prevailing separation method. Although it presents a significant number of favourable characteristics, namely, rapid extraction kinetics for many separations, the adaptability of the method to a variety of solutes and the possibility of solvent and/or diluents recycling, there are several drawbacks that must be considered. Even with the implementation of rigorous protection measures concerning operator's safety and environmental standards, most of the organic solvents still used in these extractive processes are toxic, flammable and volatile. Furthermore, when a highly selective extractant is used, the cost of the solvent system becomes very high, not to mention that sometimes large volumes of sample, extractants or organic solvents are required. Another important aspect that must be taken into consideration is that partitioning of a polar or charged solute from an aqueous phase into an organic medium requires dehydration, the extent of which depends upon the organic solvent. In some separations schemes this is trivial, while in others, particularly for the separation of metal ions, it is of paramount importance [1–3].

The exploitation of aqueous biphasic systems for the preconcentration and separation of desired analytes has gained a noteworthy importance in recent years. The aqueous biphasic systems can be formed when a certain water-soluble polymer is dissolved in water together with another kind of hydrophilic polymer or with a given inorganic salt at specific concentrations. The resulting systems are composed of two immiscible phases, which are intrinsically aqueous [2,4]. These aqueous biphasic systems have been successfully used for the separation of biological materials because they essentially have a non-denaturing environment [5]. It has also been shown that the aqueous two-phase partition technique can be suitable for the separation of inorganic compounds and small organic molecules [6–8].

Although a number of different water-soluble polymers may be utilized to form aqueous biphasic systems, polyethylene glycols (PEGs) are those primarily selected, mostly in combination with distinct inorganic salts. These liquid–liquid extraction systems have several unique advantages over traditional solvent extraction systems, such as, PEG is not expensive, it is commercially available and biodegradable, in the formation of extraction system, no addition of organic solvents is required and several inorganic anions can be used as water-soluble extractants [3,9,10]. On the basis of these considerations, the aqueous PEG–inorganic salt biphasic systems

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can be considered non-toxic, non-flammable and non-volatile, thus being friendly to the environment. In addition, in these aqueous biphasic extraction systems the partitioning takes place between two aqueous immiscible phases, and therefore the effect of solute dehydration on the extraction process is minimised. For instance, when metal ions distribute to the PEG-rich phase, only a subtle reorganization of the hydration sphere may be required, whereas in traditional solvent extraction, near-complete dehydration may be necessary [2,3].

Taking into consideration the singularities of the extraction process (mass transfer) that occurs at the interface established between two immiscible phases, the fluid management strategy associated with the concept of single interface flow analysis (SIFA) [11–13], recently proposed by our group, which relies on the establishment of an unique interface where all involved solutions are put in contact for reaction development, seems to be an attractive tool to develop an automated flow systems for carrying out continuous flow aqueous PEG–inorganic salt biphasic extraction systems. This idea was emphasised by the operational versatility of SIFA systems that enable the automated variation of the contact time between the two phases and the inter-penetration of mutual adjoining zones that could be reinforced by multiple flow reversals, which would improve the mass transfer process. Another important advantage of SIFA systems is that, since they do not rely on the utilization of well-defined and compelling sample and reagent volumes, but simply on the establishment of a unique solutions mutual inter-penetration interface, analytical system configuration is greatly simplified and enhanced operational versatility is obtained.

The aim of this work was then the implementation and evaluation of a SIFA system for carrying out the aqueous biphasic extraction of lead and subsequent on-line fluorimetric determination upon reaction with 8-hydroxyquinoline-5-sulfonic acid, the single interface acting simultaneously as extraction (enrichment) and reaction interface. To our knowledge this is the first time that aqueous biphasic systems are exploited in flow analysis.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with water from a Milli-Q system (Specific conductivity <0.1 $\mu S\,cm^{-1}$) and chemicals of analytical reagent grade quality. Reagents were not subject to any further purification.

Polyethylene glycols (PEGs) used in this study, PEG-200, PEG-400 and PEG-600, were purchased from Sigma (St. Louis, MO, USA). Working solutions of 30%, 40% and 50% (w/v) were prepared by suitable dilutions with deionised water.

The inorganic salt solutions of ammonium sulphate 30%, 40% and 50% (w/v), sodium sulphate 20% (w/v) and sodium nitrate 40% (w/v) purchased from Sigma (St. Louis, MO, USA) were prepared by dissolving of suitable quantities of solid chemical in water. pH values (3, 6, 7, 8 and 9) assayed were adjusted by using small volumes of concentrated acid or base solutions.

The 1.0×10^{-3} mol L^{-1} 8-hydroxyquinoline-5-sulfonic acid (HQSA, Sigma, St. Louis, MO, USA) solution was prepared by dissolving 56.3 mg in 250 mL of water. Other working solutions were prepared by appropriate dilution with water.

A 3.0×10^{-3} mol L^{-1} stock solution of lead (Pb(II)) was obtained by dissolving lead(II) nitrate (purchased from Sigma, St. Louis, MO, USA) in water. An intermediate solution of 2.4×10^{-4} mol L^{-1} Pb(II) was prepared by proper dilution of this stock solution. Working standard solutions were daily prepared by suitable dilutions of this intermediate solution with water.

2.2. Apparatus

The single interface flow system comprised four solenoid micro-pumps (Bio-Chem Valve Inc., Boonton, NJ, USA, 20 μL per stroke), three 161 T 031 (NResearch, West Caldwell, USA) two-way solenoid valves, and a PerkinElmer model LS-30 spectrofluorimeter equipped with a 7-μl inner volume flow cell. Reaction coils were made of PTFE tubing (0.8 mm i.d.). Homemade end-fittings, a confluence point and connectors were also used.

A computer was used for system control and for data acquisition; software was developed in Microsoft Quick-Basic 4.5. The computer was equipped with a PC-LABCard model PCL-711B (Advantech, Cincinnati, OH) interface card. A CoolDrive (NResearch Inc., West Caldwell, USA) power drive was used to operate both the solenoid micro-pumps and solenoid valves. Analytical signals were also recorded in paper using a Kipp & Zonen (Delft, The Netherlands) BD 111 strip chart recorder.

2.3. Single interface flow manifold and procedure

The developed flow manifold, pictured in Fig. 1, comprised four solenoid micro-pumps (P_1, P_2, P_3 and P_4) for inserting and propelling the sample and reagent solutions. The repetitive micro-pump switching on/off created a pulsed flowing stream in which the pulse volume corresponded to the micro-pump stroke volume. Three two-way (normally closed) solenoid valves (V_1, V_2 and V_3) were used to direct the flowing streams.

The analytical cycle was started by establishing a baseline, which was accomplished with the 8-hydroxyquinoline-5-sulfonic acid (HQSA) solution. To this end, P_4 was actuated for propelling this solution through the reaction coils C_2 and C_3 towards the detector. Subsequently, P_2 and P_3 were actuated, P_4 was switched off and the ammonium sulphate solution and the lead standard solution were inserted into the analytical path, through the reaction coils C_2 and C_1 and directed towards V_1 (Fig. 2, step A). Next, P_2 and P_3 were switched off and P_4 was actuated (two strokes corresponding to 40 μL) in order to clean the analytical path between the confluence X and solenoid valve V_2. Subsequently, P_1 was actuated, P_4 was switched off and the PEG solution was propelled through reaction coils C_1 and C_2 till V_2 (Fig. 2, step B), enabling the establishment of the single extraction interface. Finally, P_4 was actuated, P_1 was switched off and the extracted lead solution was propelled towards the detector (Fig. 2, step C). Table 1 shows the protocol sequence for SIFA aqueous biphasic extraction of lead(II). The reaction product (metal chelates) formed as a consequence of the mutual sample/HQSA intermingle produced an analytical signal, which was recorded as a peak when the reaction interface passed through the flow cell. The excitation and emission wavelengths were set at 386 and 517 nm, respectively.

3. Results and discussion

3.1. Aqueous biphase formation

Water is the main component in each phase and the dissolved components have high affinity for it. The ethylene oxide units of polyethylene glycols are known to be hydrated strongly with two to three water molecules per ethylene oxide group. The high water solubility of polyethers has, in fact, been attributed to this factor [5]. Similarly, ionic species in solution are known to be hydrated and the extent of hydration depends upon the valence of the ion. Thus, the higher the valence of the anion, the more effective in "salting-out" the PEG to form the biphasic system it is expected, because of competition for water. In the case of cations, one can expect competition between two opposing effects, namely, hydra-

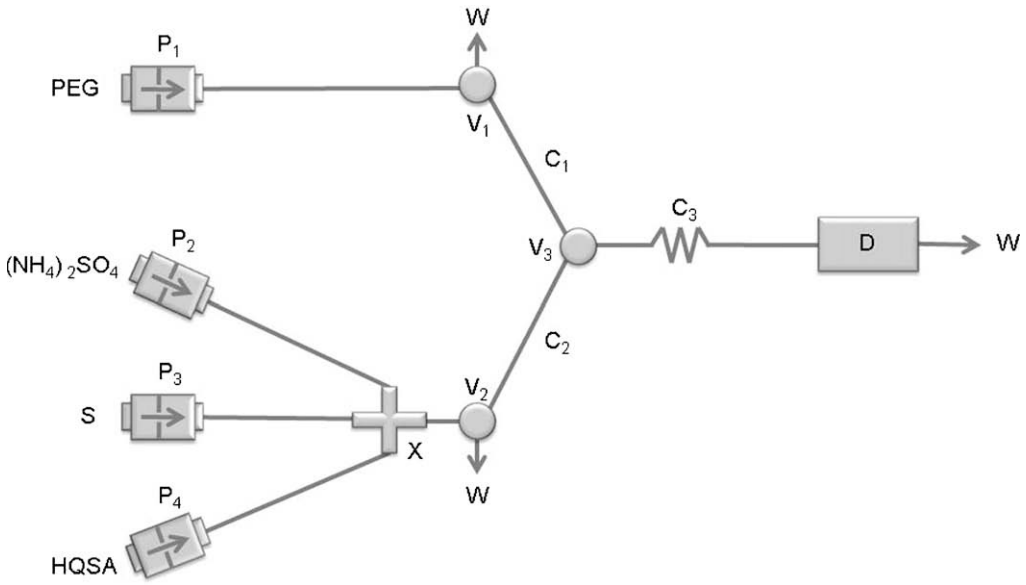

Fig. 1. Single interface flow manifold for on-line aqueous biphasic extraction of lead(II). *Legend*: P_1, P_2, P_3, P_4: solenoid micro-pumps; X: confluence; V_1, V_2, V_3: solenoid valves; C_1, C_2, C_3: 50 cm reaction coils; PEG: polyethylene glycol; S: lead(II) standard; HQSA: 8-hydroxyquinoline-5-sulfonic acid; D: spectrofluorimetric detector; W: waste.

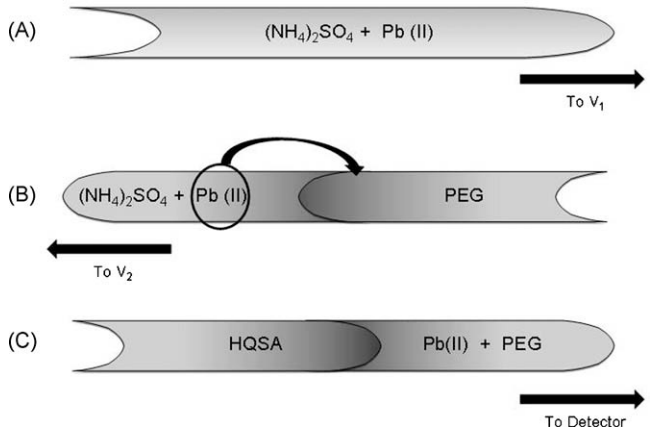

Fig. 2. Schematic representation of the extraction procedure in SIFA system.

tion and tendency to complex with polyether oxygen. Multivalent cations, in general, have been suggested to interact strongly with the ether oxygen atoms of PEG. So, it is expected that they "salt-in" rather than "salt-out" the PEG. Thus, the "salting-out" tendency of the anion is offset to some extent by the metallic cations and this can account for the apparent reversal of the effectiveness of salts to form the biphasic system [5].

The formation of aqueous biphasic systems clearly indicates the mutual exclusion of the salt and the polymer and their affinity for the solvent. Even though the PEG as a whole is non-ionic, the lone pair of electrons on the ether oxygen imparts an anionic character to the polymer and this is clearly evident from the binding of multivalent cations by polyethers. Thus a repulsive interaction between the anions and the PEG especially in the presence of nonbonding cations (such as, K^+, NH_4^+, Na^+), can occur in the system [5].

As it can be seen, the partition of an analyte ion in aqueous biphasic systems depends on several factors originating from the polymers and inorganic salts used for forming the two phases, including naturally their concentrations. For moreover, it was demonstrated that the partition coefficients follow from the relative "salting-out" ability of the salts used [4].

3.2. Development of the single interface flow methodology

In the development of the single interface flow methodology several experiments were carried out in order to improve system performance, namely in terms of enrichment factor, sensitivity, accuracy, precision and sampling rate. These features influenced the choices made during the optimisation of the systems, which was carried out using the univariate method. Since no well-defined sample or reagent volumes were used, these parameters were not subject to evaluation, thus simplifying the optimisation process.

Nonetheless, before starting the optimisation of the single interface flow methodology, a fundamental parameter had to be evaluated in order to proceed with the assessment of chemical and physical parameters: the number of pulses of PEG solution required to initiate and to propel the aqueous biphasic extraction interface back to V_2 (Fig. 2, step B), guaranteeing that the interface was not sent to waste, due to the introduction of an excessive number of PEG solution pulses nor that the number of PEG pulses was too low preventing the interface zone with the highest lead concentration to be put in contact with the HQSA reagent solution. A 40% (w/v) PEG-400 solution, 40% (w/v) $(NH_4)_2SO_4$ solution (pH 9), 2.4×10^{-5} mol L^{-1} Pb(II) standard solution and 5.0×10^{-4} mol L^{-1} HQSA solution were used. The number of pulses was evaluated from 15 to 24 pulses, corresponding to a volume of PEG between 320 and 480 μL. The results obtained in this study (Fig. 3) showed that the analytical

Table 1
Protocol sequence for SIFA aqueous biphasic extraction of lead(II).

Step	Flow rate (mL min^{-1})	Volume (μL)	Description
1	3.0	500	$(NH_4)_2SO_4$ and Lead(II) standard solution towards V_1
2	3.0	40	Clean-up of the analytical path between the confluence X and V_2 with HQSA solution
3	3.0	400	PEG solution towards V_2
4	6.0	1500	HQSA solution towards the detector

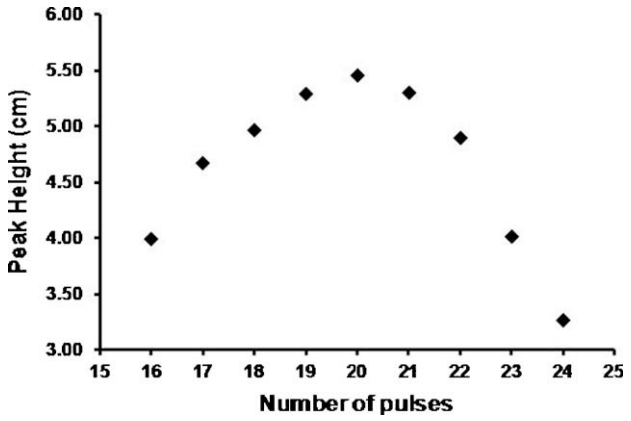

Fig. 3. Study of the number of pulses of PEG solution.

signal increased with the number of pulses up to 20 pulses (corresponding to 400 μL of PEG solution) and then markedly decreased, confirming that 20 PEG pulses are sufficient to place the interface zone with highest lead concentration in front of V_2, thus ready to react with the HQSA.

3.2.1. Chemical parameters

Solutions of PEG with distinct molecular weight (PEG-200, PEG-400 and PEG-600) were assayed in order to assess the one providing the highest extraction efficiency. A 40% (w/v) $(NH_4)_2SO_4$ solution (pH 9), 2.4×10^{-5} mol L^{-1} Pb(II) standard solution and 5.0×10^{-4} mol L^{-1} HQSA solution. Results showed that PEG-200 provided the highest analytical signal, corresponding to the greatest enrichment factor. The evaluation of the most adequate PEG-200 concentration was carried out for concentrations ranging from 30 to 50% (w/v), the results confirming that the highest analytical signal was effectively obtained for a concentration of 40%, which was selected for the experiments.

Different inorganic salts were study with the purpose of assessing their capability to provide lead(II) extraction and preconcentration into the PEG phase. These included: ammonium sulphate 40% (w/v), sodium sulphate 20% (w/v) and sodium nitrate 40% (w/v). The obtained results showed that only with ammonium sulphate a significant enrichment of lead(II) was observed. Thus, ammonium sulphate exhibits the strongest "salting-out" effect on PEG, which contributes to a more efficient extraction system. The concentration of the $(NH_4)_2SO_4$ solution was evaluated between 30 and 50% (w/v), and it was observed that a 40% (w/v) value enabled the attainment of the highest analytical signal.

The evaluation of the pH of the $(NH_4)_2SO_4$ solution showed that it had a markedly influence on the enrichment factor obtained. By assaying pH values between 3 and 9 it was observed that the solution with a pH of 9 was more suitable for this extraction process as it achieve higher analytical signals and a better enrichment of lead(II). As a result, a 40% (w/v) $(NH_4)_2SO_4$ solution with pH 9 was selected for the next assessments.

Influence of HQSA concentration was investigated between 1.0×10^{-5} and 1.0×10^{-3} mol L^{-1}. The obtained analytical signals showed a pronounced increase up to HQSA concentration values of 1.0×10^{-3} mol L^{-1} approaching stabilisation for higher values. Therefore, 1.0×10^{-3} mol L^{-1} HQSA solution was used in the subsequent experiments.

3.2.2. Physical parameters

The combined length of C_1 and C_2 is very important in terms of extraction efficiency, as it determines the residence time of the interface in the analytical system and, therefore, the extension of mass transfer between phases. We have assayed combined values of 50 cm (25 cm of C_1 + 25 cm of C_2), 100 cm (50 + 50) and 200 cm (100 + 100). The obtained results showed that the extraction efficiency increased with the combined reactor length. However, the slight increment obtained with a 200 cm reactor regarding the 100 cm one was not enough to compensate the concomitant increase in the extraction time. The 50 cm reactor provided the faster extraction but the enrichment factor was inferior. Accordingly, as a compromise between sensitivity and sampling rate we selected the 100 cm combined reactor for the succeeding experiments.

The length of the serpentine reaction coil C_3 was also important, affecting the extension of the reaction between Pb(II) and HQSA, thus the fluorescence intensity. It should be stressed out that the fluorimetric reaction occurred within C_2 and C_3. However, since C_2 was already optimised in a previous stage, in this step only the length of C_3 was subject to study. The evaluation of the analytical signals obtained with C_3 reaction coils ranging from 25 to 100 cm revealed that the analytical signal increased up to a coil length of 50 cm and then slightly decreased. Consequently, a 50 cm reaction coil was selected for further experiments (this way the combined length of C_2 and C_3 was 100 cm).

Influence of flow rate used to propel the reaction interface to the detector (Fig. 2, step C) was assessed between 2.4 and 6.0 mL min^{-1}. Increasing flow rate increased the magnitude of the analytical signal up to 3.0 mL min^{-1} and then approached stabilisation. Since a flow rate of 6.0 mL min^{-1} provided the highest sampling rate without affecting precision it was the one selected for the assays. The extraction flow rate (steps A and B, Fig. 2) was also studied between 1.7 and 6.0 mL min^{-1}. The analytical signal increased till a 3.0 mL min^{-1} flow rate and for higher flow rates the magnitude of the signal showed a pronounced decrease, which could be explained by the inferior extraction time that impaired the mass transfer process. So, for these two steps a flow rate of 3.0 mL min^{-1} was selected.

3.2.3. Analytical features

Under the optimised analytical conditions exhibited in Table 2, a linear working range for lead(II) concentrations between 4.8×10^{-7} and 2.4×10^{-5} mol L^{-1} was verified (Fig. 4). The analytical curve was typically expressed as:

$$h = 1.955C + 1.320$$

where h is the peak height (cm) and C is the lead(II) concentration (expressed in mol L^{-1}). The correlation coefficient was 0.9992. Furthermore, the repeatability was good, with relative standard deviations between 0.1 and 3.0%.

In addition, SIFA system allowed a lead(II) enrichment factor of 3. This value was achieved when comparing the analytical signals with and without the aqueous biphasic extraction procedure (steps A and B, Fig. 2).

Even considering that the unique goal of this work was the implementation of a aqueous biphasic extraction system by using a continuous flow system, in this particular case a SIFA system,

Table 2
Range of values used in dimensioning the SIFA system, and selected operating conditions for the aqueous binary extraction of lead.

Parameter	Range	Chosen value
PEG	200–600	200
PEG concentration %(w/v)	30–50	40
$(NH_4)_2SO_4$ concentration %(w/v)	30–50	40
$(NH_4)_2SO_4$ pH	3–9	9
HQSA concentration (mol L^{-1})	1.0×10^{-5}–1.0×10^{-3}	1.0×10^{-3}
Reactor length (cm)	25–100	50
Extraction flow rate (mL min^{-1})	1.7–6.0	3.0
Detector flow rate (mL min^{-1})	2.4–6.0	6.0

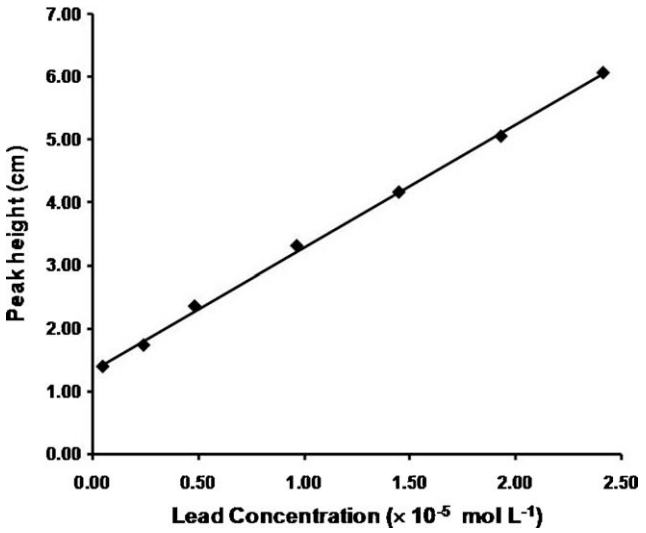

Fig. 4. Results obtained in the calibration of the system. Lead(II) standards concentrations of 4.8×10^{-7} to 2.4×10^{-5} mol L^{-1}.

and anticipating the possibility of development of an analytical methodology for monitoring trace lead in real samples, we have carried out the evaluation of the interfering effect of common metal species such as Tl, Cd, Cu, Fe, Co and Zn. It was verified that the used fluorophore assured a good selectivity for Pb, with only a significant interfering effect from zinc.

4. Conclusions

This work demonstrated the feasibility of using a single interface flow system for carrying out continuous flow aqueous biphasic extraction. The obtained results could be considered very satisfactory because this type of extraction was for the first time exploited in flow analysis and no previous data was available, the developed approach could be considered more environmentally friendly than those resorting to organic solvents and, at the same time, anticipates the possibility of coupling this extraction procedure with other detection techniques, and its application in the determination of other analytes.

Furthermore, the synergy arising from the combination of some important characteristics of continuous flow analysis, such as high determination rates, improved analytical efficiency and minor operator intervention, with the singularities of single interface flow concept, and the noteworthy advantageous of aqueous biphasic extractions makes this work an attractive and promising starting point for future trends and developments.

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CAPÍTULO 7

Determinação da carência química em oxigénio em análise em fluxo: uma revisão crítica

Determinação quimioluminométrica da carência química em oxigénio através de fotocatálise assistida por *Quantum Dots*, utilizando um sistema de fluxo com interface única

A carência química em oxigénio (CQO) é definida como a quantidade de oxigénio consumida para a oxidação química matéria orgânica presente numa amostra líquida, tornando-se, desta forma, um parâmetro fundamental para a monitorização da qualidade da água no que concerne a avaliação do efeito de agentes poluentes.

O método oficial para a determinação de CQO sujeita a amostra a oxidação quer pelo permanganato de potássio ou pelo dicromato de potássio, sob condições bem definidas. No entanto, apresenta algumas desvantagens, nomeadamente, a análise é bastante morosa (2 a 4 horas só para digerir a amostra), requer procedimentos manuais consideráveis, o que aumenta a probabilidade de erros do operador, e um consumo relativamente elevado de reagentes dispendiosos (Ag_2SO_4) e tóxicos ($HgSO_4$, $K_2Cr_2O_7$), bem como, de efluentes tóxicos gerados são verificados.

Para ultrapassar estes inconvenientes, vários métodos de fluxo foram propostos e, apesar de reduzirem drasticamente o tempo de análise, a maioria continua a fazer uso dos reagentes supra-mencionados para promover a oxidação da matéria orgânica. Simultaneamente, diferentes estratégias de digestão foram sendo investigadas com estes reagentes, incluindo micro-ondas e irradiação UV. Assim, foi elaborado um artigo de revisão onde são descritos e discutidos os vários métodos de fluxo desenvolvidos e estratégias de digestão da matéria orgânica presente nas amostras, que foram sendo implementados ao longo dos anos, observando-se por parte dos autores uma procura constante em superar as várias limitações existentes através da aplicação de novas ideias e conceitos.

Neste seguimento, foi desenvolvido um novo método para a determinação de CQO, que se baseia na combinação de um sistema SIFA, de uma unidade foto-catalítica UV e de nanotecnologia *quantum dot* (QD). A abordagem desenvolvida utiliza a capacidade dos nanocristais de CdTe para gerar espécies oxidantes, após irradiação com luz UV, que permitem a rápida degradação catalítica de compostos orgânicos. O luminol foi utilizado como sonda quimioluminescente para a avaliação indirecta de CQO, uma vez que é facilmente oxidado pelas espécies oxidantes geradas durante a irradiação dos *quantum dots* originando uma forte emissão quimioluminescente que é suprimida na presença de matéria orgânica.

O método proposto permitiu a determinação de CQO com concentrações compreendidas entre 1 e 35 mg L^{-1} com uma frequência de amostragem de 33 h^{-1}. O sistema SIFA desenvolvido foi aplicado à determinação de CQO em material de

referência certificado e os resultados obtidos mostram uma excelente concordância com os valores certificados. Para além disso, esta abordagem permite obviar vários problemas associados com a metodologia oficial para a determinação de CQO, nomeadamente, longo tempo de análise, operações fastidiosas e consumo de reagentes dispendiosos e tóxicos.

CHEMICAL OXYGEN DEMAND DETERMINATION IN FLOW ANALYSIS:

A CRITICAL REVIEW

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Abstract

A critical review that covers the description and analysis of flow methodologies developed in the past 30 years for the determination of chemical oxygen demand is presented in this work. Since the first publication, many efforts were made to overcome several concerns about the flow methodology itself and, specially, the way to attain a proper oxidation of the organic matter present in the sample. The ultimate aspiration is to create an alternative, rapid and environmentally friendly methodology for chemical oxygen demand assessment and, with the recent advances of nanotechnology associated with photocatalysis, a novel concept is foreseen.

Keywords: Chemical oxygen demand; flow analysis; spectrophotometry; electrochemistry; chemiluminescence; photocatalysis.

1. Introduction

Chemical oxygen demand (COD) is defined as the amount of specific oxidant that reacts with the sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of oxygen equivalence. Both organic and inorganic components of a sample are subject to oxidation, but in most cases the organic component predominates and is of the greater interest [1]. Therefore, COD, which represents the oxygen required for complete chemical oxidation of a water sample, is a key parameter for monitoring water quality upon assessment of the effect of polluting agents on the oxygen level.

In the official method, potassium dichromate in acidic solution is used as the oxidant. The procedure includes the sample reflux for 2 hours in strong sulfuric acid solution with a known excess of potassium dichromate. In the oxidation of the sample, dichromate is reduced to a chromic ion state. After the oxidation, the remaining unreduced dichromate, or the reduced Cr^{3+}, is measured either by titration or spectrophotometrically. This oxidation is catalysed with silver nitrate. Chloride is often the most significant source of interference and additionally it precipitates the silver catalyst. To eliminate chloride interference, mercury (II) sulfate is currently used, forming mercuric chloride complex. COD is a defined test; the extent of sample oxidation can be affected by digestion time, reagent strength, and sample COD concentration [1]. Moreover, in a COD analysis, hazardous wastes of mercury, hexavalent chromium, silver, and acids are generated.

To overcome these drawbacks several flow methodologies that reduce drastically the analysis time, and different strategies for the oxidation of the sample were investigated, which are described and discussed in detail in the subsequent sections.

2. Chemical oxygen demand detection techniques

Since 1980 almost 40 articles exploiting chemical oxygen demand determination in flow systems have been published (**Figure 1**). The type of detection technique employed in 56.8% of the publications was spectrophotometry, followed by 27% for electrochemistry and 16.2% for chemiluminescence.

2.1. Spectrophotometry

Several publications concerning flow injection analysis (FIA) for the determination of COD that involved spectrophotometric detection are listed in **Table 1**. The first flow injection strategies developed were very simple (**Figure 2A**), and they used acidic potassium permanganate solution as both an oxidant and a spectrophotometric reagent, thus replacing potassium dichromate [2-8, 16, 18, 19, 22]. The substitution of Cr (VI) by Mn (VII) from potassium permanganate originated a less toxic analysis waste, which is environmentally friendly.

The manifold included a thermostatic bath that potentiated the digestion of organic matter present in the sample. As a result, the redox reaction can occur smoothly because the temperature of the reaction manifold can easily be controlled using the thermostatic bath. This thermostatic bath was composed of water [2-5, 16, 18, 22], PEG 400 [6] or corn oil [7, 8] as heat medium. Due to the high temperature and a quite long residence time, bubbles are formed, which interfered with COD determinations [6]. Therefore, to circumvent this problem a narrow outlet (0.25 mm i.d. PTFE tubing) was fitted to the flow cell, thus creating a back-pressure of a few atmospheres in the system [4-8].

A segmented flow injection system was also developed using a special valve designed to inject the samples and the bubbles into the carrier stream in a quantitative manner. Since the sample is sandwiched between two bubbles, the dispersion is limited and the flow-rate is also stabilized [13].

Various simple organic compounds were tested as standard substances for COD, and as it can be observed in **Table 1**, the majority of the publications use glucose. However, it should be noted that glucose is not always the best standard substance for COD because, in an actual COD determination, many organic substances are much less readily oxidized by acidic permanganate solution than is glucose [6].

So, in order to evaluate a standard substance for COD, forty-five organic compounds have been oxidized with an acidic permanganate solution under various oxidation conditions. The compounds used were classified into three groups according to the oxidation values obtained by the standard method [22]. As a standard substance for COD, authors established that a mixture of the compounds has to be properly chosen according to the oxidation behavior of the water sample.

Another aspect that must be considered is the formation of a black precipitate (manganese dioxide), which is produced by the redox reaction between the acidic

potassium permanganate solution and the organic COD substances. This precipitate blocked the flow-lines especially at the narrow points of the flow system. Therefore, some strategies were implemented to prevent it, such as the utilization of different types of connectors that reduced the amount of base-line drift and noise, which had been caused by diameter variations and blockage of the flow-line, addition of ammonium sulfate to the sulfuric acid solution and also the optimization of sulfuric acid concentration [4, 6, 18].

Additionally, in a FIA system, continuous forward flow is used to transport the injected sample zone, to mix it with reagents and to carry the reaction product into a flow cell for detection. This permits accurate determination and higher sampling frequency, but consumes more reagents and generates more waste as the result of continuous pumping. To suppress the reagent consumption and to minimize the hazardous and toxic wastes, an effective solution was the adoption of a reversible system by circulating the oxidant solution in a cyclic FIA [21]. Accordingly, the acidic KMnO4 method was carried out by implementing a single-line circulating flow system. The oxidant consumed by the oxidation of organic substances was regenerated and reused repeatedly, resulting in an extreme reduction of hazardous wastes. However, the obtained COD values with the proposed method were co-related with those provided by the official standard method, but were quite low owing to the insufficient digestion step.

Some authors support potassium dichromate should still be used as an oxidizing and spectrophotometric reagent for the COD determination by the FIA method instead of potassium permanganate [9-11], given that not only the redox reaction with permanganate was essentially incomplete and was variable for the COD determination, but also, the FIA method was fairly troublesome for a continuous operation of the apparatus because of the manganese dioxide precipitate formation. Besides, in the method with dichromate, Cr (VI) is quantitatively reduced to Cr (III) by the organic substances, and therefore forms no precipitate in the tube. Hence, the FIA apparatus could be composed of a longer PTFE tubing as the reaction tube, and its continuous operation could easily be achieved for a long time. Moreover, no contamination by organic substances occurred in the tubes of this FIA system because the acidic dichromate solution as a strong oxidant constantly flowed into them [9].

Furthermore, there is a large difference between digestion time in the conventional methods (2 h) and in the flow methods (a few min). This also means that the amounts of energy applied in both cases are very different and, hence, that in some cases oxidation of the organic matter may not be complete using only the thermostatic bath in a FIA system. For this reason, more efficient heating methods have been applied, microwave

radiation among them. Owing to the heating mechanism (i.e., dipole rotation and ion migration), microwave heating is much more effective and rapid than conventional (i.e., conductive and convective) heating [12, 15, 17].

There were developed some FIA systems with a microwave included in the manifold (**Figure 2B**), in order to potentiate the digestion of the organic matter present in the sample [12]. After leaving the microwave oven, the flow enters a gas diffusion unit to remove gas bubbles [12] or into an ice bath [15, 17] to cool the digested sample and to condensate any vapours, that might have been produced in the digestion step.

Afterwards, if the COD should be determined by flame atomic absorption spectrophotometry, the sample could flow through an anionic exchange resin that retains the Cr (VI) that has not been reduced by the organic matter of the sample, since the main problem is that Cr (III) and Cr (VI) need to be separated before reaching the spectrophotometer (speciation). Then, the retained Cr (VI) could be eluted and directed towards the detector. Moreover, interferences due to the matrix nature are absent, since matrix is washed out of the column before Cr (VI) is eluted [15].

However, this procedure includes a separation step that requires the presence of a skilled analyst. Furthermore, the resin deteriorates quite rapidly due to the high acidity of medium. These problems make this method slightly inconvenient to use.

Hence, a new procedure for separating chromium species was proposed in order to make handling easier. It consists in a liquid-liquid extraction of Cr (VI) with tributyl phosphate after the digestion step in the microwave. The remaining (non-reduced) Cr (VI) is determined in the organic phase also by atomic absorption spectrophotometry [17].

Nonetheless, the main difficulty in using microwave oven is that the energy is not supplied in a continuous way but in a pulsed way, the rhythm being a function of the present total power output. In a FIA system, where the volume of the solutions flowing into the reaction coils is very small, this means having a series of “microboiling” steps with a visible expansion of the solution volume in the tubes, alternating with a series of “cooling steps”, controlled by the magnetron-generated pulses. The result is a very consistent production of bubbles and the impossibility of making any kind of measurement with a spectrophotometric detector unless an adequate and efficient debubbling system is used [12].

Furthermore, as it can be perceived, the main problems still to be resolved are the toxic effluent produced during the COD determination and the complicated operating

conditions necessary to obtain a high degree of sample oxidation. Some of the FIA systems proposed still make use of silver as a catalyst [2, 8, 10, 11, 15, 17] and mercury (II) to eliminate interferences [10, 11, 15, 17].

Accordingly, a FIA method was proposed using a thermostatic bath and Ce (IV) as the oxidizing reagent, since Ce (IV) is not only non-toxic but also has a higher redox potential than Mn (VII) and Cr (VI) [14]. In addition, it does not need mercury (II) to prevent chloride interference under 30 g L^{-1}, because the use of low acid concentration inhibits the reactions of the interfering compounds with oxidizing agents. Owing to the low acid concentration used, other two problems were solved: the reaction can proceed without the formation of bubbles and the temperature reaction could be controlled, because in normal conditions merely mixing the reagent solutions and carrier streams generates a stream temperature of about 100ºC even without heating, and thus precise control of reaction temperatures becomes difficult.

This method gained interest on continuous monitoring for diverse applications such as process control of wastewater treatment. Unfortunately, the technique does not lend itself to continuous monitoring when unattended over a long period, because the gradual self-decomposition of cerium (IV) sulfate solution may be more rapid than expected and its concentration should be checked every other day. Its self-decomposition results in a base-line drift and a gradual variation of the degree of sample oxidation. This problem remains to be resolved [14].

Finally, another approach exploited the UV photocatalytic oxidation of organic compounds in a FIA system (**Figure 2C**) instead of conventional heating, with acidified potassium permanganate as the oxidant [20]. UV photocatalysis is an efficient alternative to wet digestion for the degradation and mineralization of organic compounds and eliminates the need for high temperature oxidation, significantly reducing the required digestion time.

Preliminary studies conducted with a 400 W medium pressure UV lamp resulted in the precipitation of manganese dioxide in the photo-reactor coil and a noisy, drifting baseline. The use of a 15 W low pressure UV lamp minimized the precipitation of manganese dioxide, significantly reduced the noise and stabilized the baseline. Furthermore, in common with other COD methods, the proposed method suffered from chloride interference which was oxidized by the permanganate, resulting in a positive interference [20].

In fact, spectrophotometry is the most common detector used in COD determination. However, there are some inherent problems of these systems with spectrophotometric measurements, such as, in order to increase the oxidizing ability of reagents and decrease the digestion time, these systems are heated so as to produce bubbles, and the signals are frequently unstable due to bubble formation; samples showing high turbidity are not suitable for this method due to serious interference; the systems lack enough sensitivity so that the systems need rather long digestion time and are not suitable for the samples with low COD.

2.2 Electrochemistry

In recent years, much attention has been focussed on the photocatalytic decomposition of organic pollutants in water. The investigations have proved that many semiconductors in aqueous solution, under light irradiation and with higher energy than that of the band gap of the semiconductor, will induce catalytic decomposition and often complete mineralization of organic compounds [23].

A semiconductor particle is believed to have a filled valence band separated from a vacant conduction band by a gap whose energy is E_g. When irradiated with a light source, whose energy exceeds E_g, an electron is promoted from the valence to the conduction band, leaving behind a positive hole. Upon migration to the surface, the electron would reduce any available species. In contrast, the hole would react with water to produce hydroxyl radicals (**Figure 3**). These radicals play an important role in oxidizing organic pollutants [24].

Several photocatalytic processes use the semiconductor titanium dioxide (TiO_2) as a photocatalyst because of its distinct characteristics, such as non-photocorrosive, non-toxic, and ability for the photooxidative destruction of most organic pollutants.

Hence, a photocatalytic sensor for the determination of COD with flow injection analysis was proposed in several publications [23-26]. This sensor is based on the photocatalysis of organic compounds in the presence of TiO_2 beads in a photochemical column (**Figure 4**). The sensor was developed in conjunction with TiO_2 beads in the photochemical column and with an oxygen electrode as the sensing part. The sensor signal was observed as a result of the detection of dissolved oxygen changes due to photocatalytic oxidation of organic compounds in the sample solution.

The main advantages of this photocatalytic sensor for COD is its simplicity of preparation, low cost in the manufacturing process for the sensor, high oxidative ability and safety, fast response time, acceptable lifetime, and potential for automated monitoring [24]. Nevertheless, its application in COD determination was seldom reported, probably due to its narrow linear scope and liability to be influenced by the reductive or oxidative substances presented in the sample, such as O_2 and chloride [26].

Another approach was the utilization of a lead dioxide (PbO_2) electrode for the electrocatalytic oxidation of the organic matter. Even so, its utilization was restricted since it is incapable of degrading various kinds of organic pollutants.

In order to overcome this shortcoming, an electrochemical detection system with a PbO_2 modified electrode for flow injection analysis was then suggested [27, 28]. Doping with F^- can increase the amount of the unoccupied surface sites, which improved the electrocatalytic ability of PbO_2 anode. When the F-PbO_2 electrode generates HO• radicals, organic compounds are electrocatalytically oxidized by the unoccupied surface sites in a thin-layer amperometric detector. The COD value is proportional to the current response of the working electrode [27].

To enhance even more the oxidation efficiency and to minimize the degradation time, a Ti/TiO_2 electrode [29] and a Ti/TiO_2/PbO_2 electrode [30] were developed for the photoelectrochemical catalytic degradation of organic matter that was also carried out in a thin-layer amperometric detector. The catalytic activity of Ti/TiO_2/PbO_2 electrode was improved greatly because both photocatalysis and electrocatalysis were carried out at the same time and they were synergistic [30]. This type of approach also avoids the problems associated with the use of oxygen as electron scavenger in the methods based on TiO_2.

A voltammetric electronic tongue with automated operation based on FIA technique was applied to the characterization of wastewaters coming from the paper mill industry [31]. A metallic multielectrode array (formed by platinum, gold and rhodium electrodes) was developed as the detection system. The obtained results do not allow using the method as it stands for COD measurements, but it seems a promising approach to a quick and simultaneous assessment of COD, conductivity and pH.

Recently, a boron-doped diamond (BDD) electrode as a detecting element was implemented for the amperometric determination of COD in a FIA system [32]. BDD is a versatile, environmentally friendly electrode material that has been widely studied in the fields of electrochemical water treatment and electrochemical analysis. The BDD material possesses many interesting properties such as wide working potential window, low

background current, long term response stability, high mechanical strength, and corrosion resistance. Moreover, the combination of a FIA system and a BDD electrode promoted the development of an environmentally friendly, *in situ* and on-line COD testing system.

Table 2 presents a brief summary of the main features of these publications involving electrochemical detection. Indeed, electrocatalytic oxidation presents many advantages as the rapidity of the analysis, the directness in the acquisition of analytical signal, and the ease to be incorporated into an on-line analysis monitoring system. However, the reliability of these new methods should be further improved to satisfy practical use. For instance, a COD sensor has many benefits as it was shown, including also no need for any sample pre-treatment, great potential for *in situ* and real-time monitoring. Nevertheless, this approach still exhibit low sensitivity caused by the small change of dissolved oxygen during the degradation and inadequate oxidation due to short analysis time.

2.3. Chemiluminescence

Up to now, not many chemiluminescence methods in flow analysis for determining COD were reported (**Table 3**), although it is known to be a powerful analytical technique that promises high sensitivity, fast response time, extensive dynamic range, and use of simple and inexpensive of instrumentation easy to set up a rapid reproducible means of detection.

One of the first publications developed a system where potassium permanganate was reduced to Mn^{2+} [33]. This specie was first adsorbed on a strongly acid cation-exchange resin mini-column to be concentrated during chemical oxidation of the organic compounds at room temperature, while the excessive MnO_4^- passed through the mini-column to waste. Then the concentrated Mn^{2+} was eluted reversely and measured by the luminol-H_2O_2 chemiluminescence system. Since this system is very sensitive for Mn^{2+}, it is not necessary a long digestion time and heating the system. Moreover, the oxidation between luminol and H_2O_2 is very slow in absence of catalyst at the room temperature, thus these two reagents were mixed as the luminescence reagent.

In this system, the interference of chloride ion is slight. Some of the main reasons rely in the short reaction time between oxidizing reagent and sample that could not be enough for development of the side reaction of the reagent with chloride ion, and without heating, $KMnO_4$ oxidized slowly the sample at room temperature and very small amount of

chloride ion could be oxidized. Another drawback is that the chemiluminescence method lacks selectivity, given that other metals interfere with the determination of Mn^{2+}. To solve this problem, another cation ion-exchange column was wisely introduced into the sample stream.

A following publication, achieved also satisfactory results based only on $KMnO_4$-luminol reaction. In this case it was not used H_2O_2 and the cation-exchange resin was substituted for EDTA that avoided successfully the interfering effects caused by metal ions [34].

In another FIA system, potassium dichromate was used instead of permanganate. Dichromate was reduced to Cr^{3+} in H_2SO_4 during the chemical oxidation of COD substances in the sample, and then Cr^{3+} was measured with a luminol-H_2O_2 chemiluminescence system. The interfering effects of coexisting metal ions were masked also with EDTA, since the formation of the Cr(III)–EDTA complex is kinetically slow [35].

Although these mentioned systems do not make use of silver or mercury (II), they still employ dichromate or permanganate.

Research on an advanced oxidation process demonstrates that the basic requirement for the candidate oxidant is its ability to rapidly oxidize all contamination. Usually, combining several reagents provide a higher oxidation efficiency when compared with individual ones. In the case of a certain organic compound that can hardly be degraded by ozonation or photolysis alone; if it was subjected to the photocatalytic effects of UV irradiation, it would enhance significantly the ozonation reaction efficiency.

Hence, it was proposed a chemiluminescence FIA method for the determination of COD in which ozone was used as the oxidant assisted by UV radiation. It was based on the phenomenon that luminol can be oxidized by the dissolved ozone and other free radicals to produce luminescence [36].

It should be noticed that in aqueous solution, the radicals which did not react with the sample will interfere with the measurement and, to prevent it, $NaHCO_3$ was applied as a scavenger of free radicals. In addition, trace metal ions, can also catalyze the process of luminol oxidation, so Co as a catalyser whose concentration is several hundred times higher than that of trace metals in the matrix was added.

Based only in the fact that a quantitative amount of free radicals can be produced by analytes in the UV irradiation process and in the same phenomenon described previously for luminol, COD was also successfully determined indirectly [37]. The

proposed method is not only environmentally friendly (since it does not require any strong oxidizing reagent and any catalysts such as TiO_2) but also very simple FIA system (mainly with a photo-reactor and a chemiluminescence detector). In addition, unlike traditional COD methods that use dichromate or permanganate, chloride ion does not significantly interfere with the measurement even when its concentration is as high as 10000 mg L^{-1}. Regarding metal ions interference, it can be eliminated simply by diluting the sample owing to its high sensitivity nature, or by introducing a cation ion-exchange column into the sampling stream. Even though this method presents several advantages, it is not able for the degradation of some organic compounds, as assumed by the authors, for instance, potassium hydrogen phthalate (KHP) – a common COD standard – can hardly be degraded completely under the conditions in this work.

At last, for the first time, CdTe quantum dots (QDs) were used to promote the photocatalysis of several organic compounds upon irradiation with UV light, which was used as a means to assess sample COD [38]. QDs are nanometer-scale semiconductor crystals and are defined as particles with physical dimensions smaller than the exciton Bohr radius. The QD core is made up of elements from the II–VI (e.g. CdSe, CdTe, CdS, ZnSe), III–V (e.g. InP, InAs) or IV–VI (e.g. PbSe) group [39].

To carry this approach, a fully automated single interface flow system (SIFA) [40] comprising a photo-irradiation unit was developed (**Figure 5**). The proposed approach takes advantage of the CdTe quantum dots capacity to generate oxidizing species when irradiated with UV light thus promoting the photocatalysis of organic compounds. The implemented analytical system allowed the monitoring of the light emitted upon oxidation of luminol by the oxidizing species generated during CdTe quantum dots irradiation.

This work introduced in flow analysis a novel approach for COD determination. Coupling the concept of photocatalysis to a fully automated SIFA system, for the first time, by the incorporation of on-line UV irradiation was a challenge that demonstrated the feasibility of this type of flow methodology. Moreover, CdTe quantum dots (in aqueous medium) were used, also for the first time, for the COD determination, owing to its unique characteristics as a semiconductor, that upon irradiation with UV light they assisted the photocatalysis of organic compounds.

3. Conclusions

Despite its widespread use, the official method for COD determination has several disadvantages such as: (i) the analysis is time-consuming (2–4 h being required for digestion plus additional time for the titration); (ii) handling is also considerable, increasing the probability of errors; (iii) a relatively high consumption of expensive (Ag_2SO_4) and toxic ($HgSO_4$, $K_2Cr_2O_7$) chemicals is verified.

To overcome these drawbacks several flow methodologies with different detection techniques (spectrophotometry, electrochemical and chemiluminescence) were developed. Although they reduce drastically the analysis time, most of them still used the supra mentioned chemicals for promoting the oxidation of the organic matter. At the same time, different digestion strategies were proposed with these chemicals, including microwave and UV irradiation.

In recent years, the photocatalytic decomposition of organic pollutants in water has received much attention and started to be considered as an environmentally friendly alternative for COD assessment. Moreover, recent investigations have proved that many semiconductors in aqueous solution induce catalytic decomposition and often complete mineralization of organic compounds. Therefore, as it can be forecasted, allying nanotechnology with photocatalysis in flow analysis makes it an attractive and promising start point for future trends and developments.

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Figures:

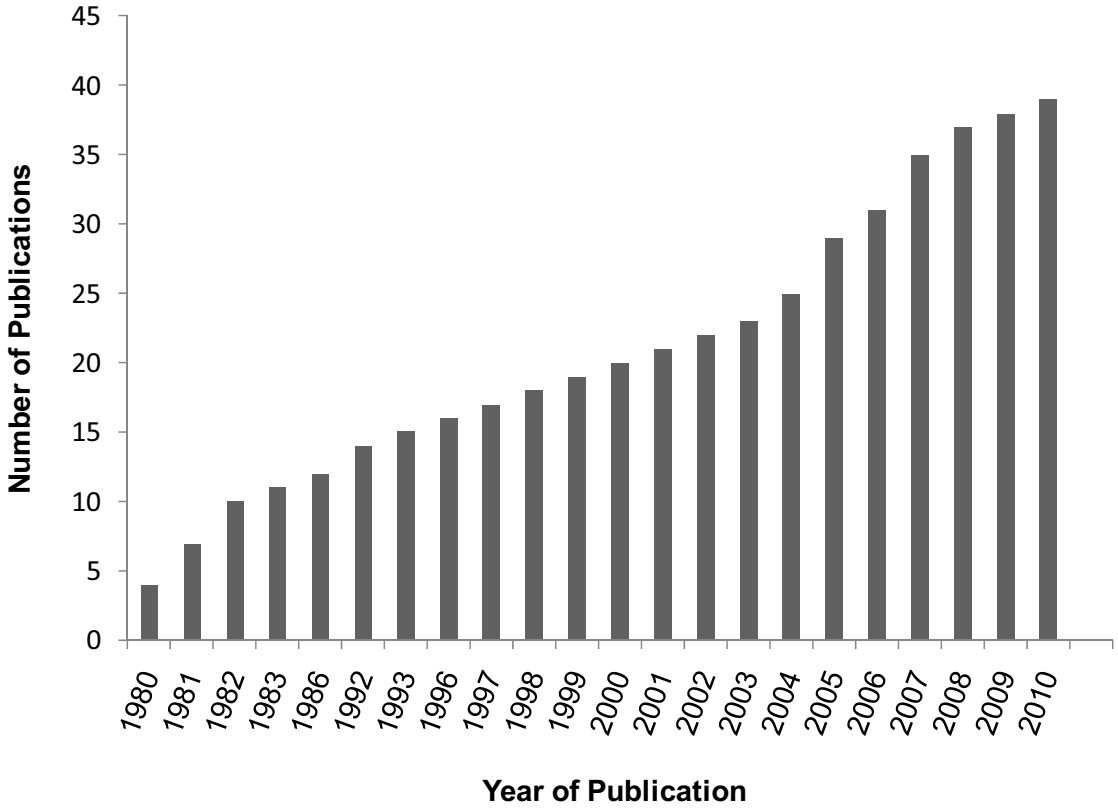

Figure 1 – Annual distribution of publications based on COD determination in flow analysis.

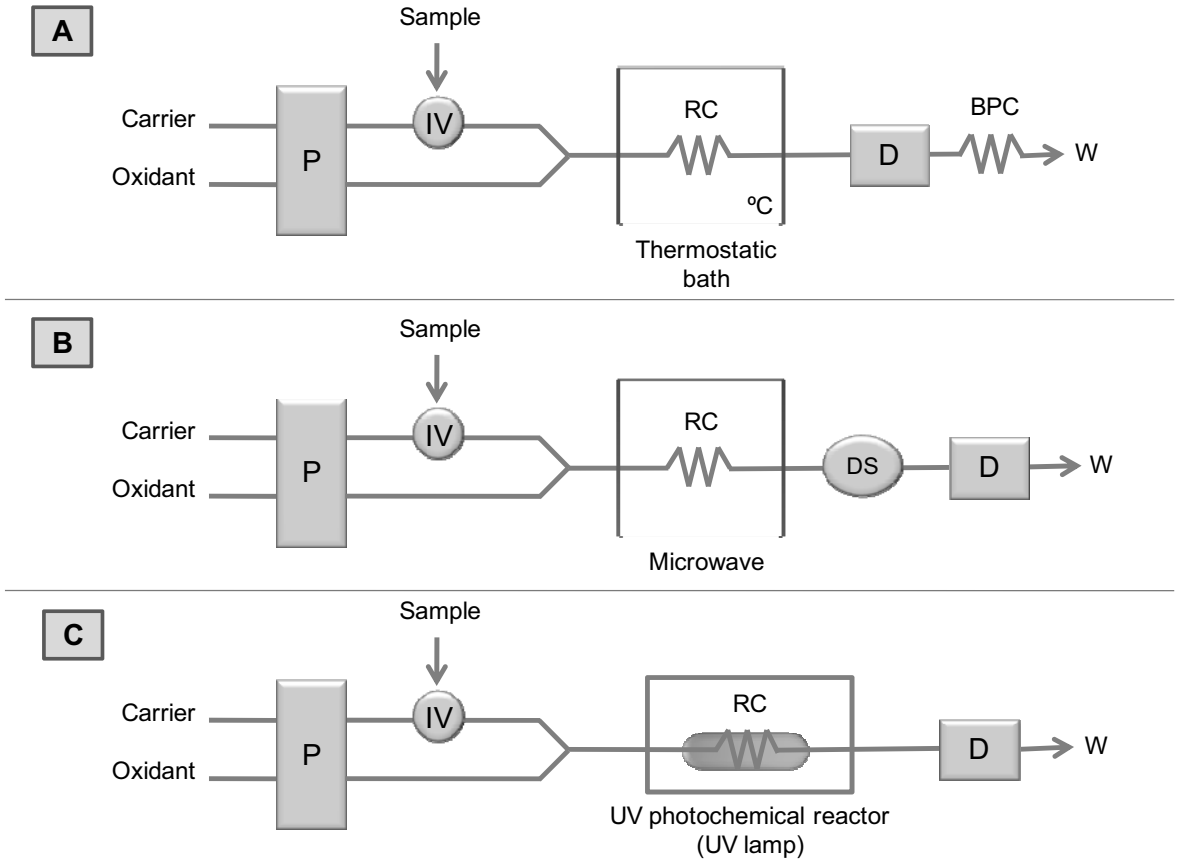

Figure 2 – Flow injection manifold for the spectrophotometric determination of COD with different digestion techniques: (A) thermostatic bath, (B) microwave, (C) UV irradiation. Legend: P: peristaltic pump; IV: injection valve; RC: reaction coil; D: detector; BPC: back-pressure coil; DS: debbubling system - condenser (ice bath) or gas diffusion unit.

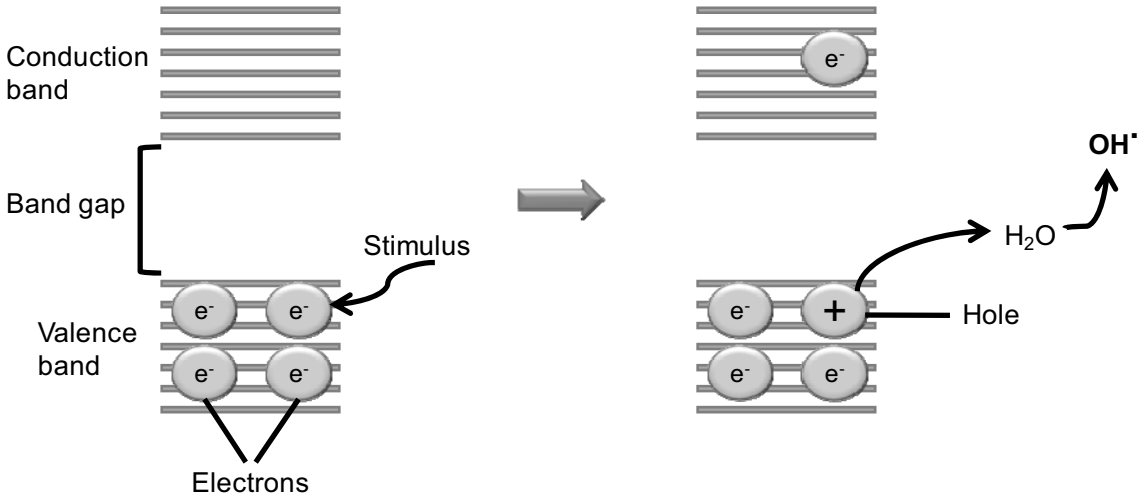

Figure 3 – Schematic representation of the production of hydroxyl radicals in a semiconductor.

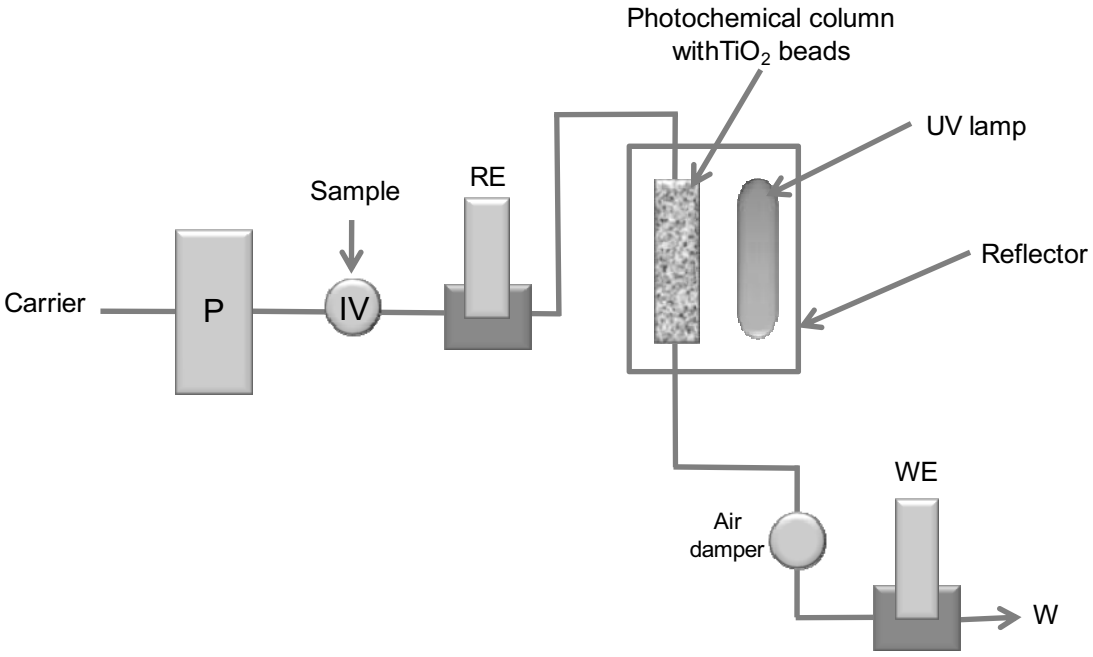

Figure 4 – Flow injection manifold for the photocatalytical electrochemical determination of COD. Legend: P: peristaltic pump; IV: injection valve; RE: reference electrode; WE: working oxygen electrode.

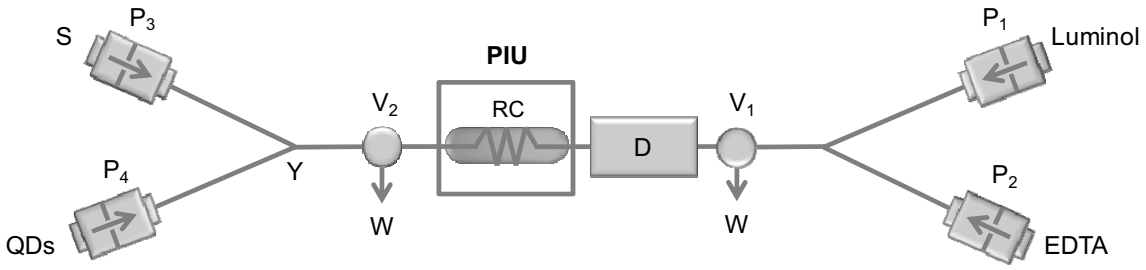

Figure 5 – Single Interface Flow Manifold for the determination of COD. Legend: P_1, P_2, P_3, P_4: solenoid micro-pumps (10 μL per stroke); X, Y: confluences; V_1, V_2: solenoid valves; PIU: photo-irradiation unit (15-W quartz UV lamp, λ_{max} = 256 nm), RC: 50-cm reaction coil; S: COD standard (KHP) or sample; D: chemiluminescence detector; W: waste.

Table 1 – Publications that exploit flow analysis COD determination with spectrometric detection.

COD standard	Reagents	Digestion	Sample volume (μL)	Sampling rate (h^{-1})	Linear Range	Detection limit	Reference
$Na_2C_2O_4$	$KMnO_4$ in H_2SO_4, $AgNO_3$	Heat, 100ºC	20	120	21 – 170 mg L^{-1}	-	[2]
$C_6H_{12}O_6$	$KMnO_4$ in H_2SO_4	Heat, 100ºC	20	20	Up to 170 mg L^{-1}	10 mg L^{-1}	[3]
$C_6H_{12}O_6$	$KMnO_4$ in H_2SO_4	Heat, 100ºC	20	30	10 – 200 mg L^{-1}	-	[4]
$C_6H_{12}O_6$	$KMnO_4$ in H_2SO_4	Heat, 100ºC	20	30	24 – 220 mg L^{-1}	10 mg L^{-1}	[5]
$C_6H_{12}O_6$	$KMnO_4$ in H_2SO_4	Heat, 100ºC	20	20	13 – 170 mg L^{-1}	5 mg L^{-1}	[6]
$C_6H_{12}O_6$, $C_{12}H_{22}O_{11}$, $Na_2C_2O_4$	$KMnO_4$ in H_2SO_4	Heat, 100ºC	30	8	5 - 200 mg L^{-1}	-	[7]
$C_6H_{12}O_6$, $C_{12}H_{22}O_{11}$	$KMnO_4$ in H_2SO_4, $AgNO_3$	Heat, 100ºC	20	-	Up to 200 mg L^{-1}	-	[8]
$C_6H_{12}O_6$	$K_2Cr_2O_7$ in H_2SO_4	Heat, 100ºC	10 - 100	10	50 – 10000 mg L^{-1}	50 mg L^{-1}	[9]
$C_6H_{12}O_6$	$K_2Cr_2O_7$ in H_2SO_4	Heat, 120ºC	100	15	Up to 160 mg L^{-1}	5 mg L^{-1}	[10]
$C_6H_{12}O_6$, KHP, $Na_2C_2O_4$, $C_7H_5NaO_3$	$K_2Cr_2O_7$ in H_2SO_4, Ag_2SO_4, $HgSO_4$	Heat, 160ºC	110	6	80 – 400 mg L^{-1}	-	[11]
KHP	$K_2Cr_2O_7$ in H_2SO_4	Microwave	250	-	Up to 100 mg L^{-1}	1.5 mg L^{-1}	[12]
$C_6H_{12}O_6$	$KMnO_4$ in H_2SO_4	Heat, 95ºC	20	80	2.0 – 80 mg L^{-1}	2.0 mg L^{-1}	[13]
$C_5H_9NO_4$, $C_{12}H_{22}O_{11}$	$Ce(SO_4)_2$ in H_2SO_4	Heat, 100ºC	50	20	0.5 – 130 mg L^{-1}	0.5 mg L^{-1}	[14]
KHP	$K_2Cr_2O_7$ in H_2SO_4, Ag_2SO_4, $HgSO_4$	Microwave	10 - 240	Up to 10	25 – 5000 mg L^{-1}	7 mg L^{-1}	[15]
$Na_2C_2O_4$	$KMnO_4$ in H_2SO_4	Heat, 40ºC	1000	20	0.2 – 18.2 mg L^{-1}	0.13 mg L^{-1}	[16]
KHP	$K_2Cr_2O_7$ in H_2SO_4, Ag_2SO_4, $HgSO_4$	Microwave	120	9	60 – 12000 mg L^{-1}	13.5 mg L^{-1}	[17]
$Na_2C_2O_4$	$KMnO_4$ in H_2SO_4	Heat, 50ºC or 80ºC	500	18	0.1 – 5.9 mg L^{-1}	0.08 mg L^{-1}	[18]
$C_6H_{12}O_6$	$KMnO_4$ in H_2SO_4	Heat, 95ºC	-	-	Up to 100 mg L^{-1}	0.01 mg L^{-1}	[19]
$C_6H_{12}O_6$, KHP, $Na_2C_2O_4$	$KMnO_4$ in H_2SO_4	UV irradiation	100	30	0.5 – 50 mg L^{-1}	0.5 mg L^{-1}	[20]
$C_6H_{12}O_6$, $Na_2C_2O_4$	$KMnO_4$ in H_2SO_4	Heat, 70ºC or 130ºC	20	30	1.0 – 24 mg L^{-1}	0.5 mg L^{-1}	[21]

$C_6H_{12}O_6$: glucose; $C_5H_9NO_4$: glutamic acid; $C_{12}H_{22}O_{11}$: lactose; KHP: potassium hydrogen phtalate; $Na_2C_2O_4$: sodium oxalate; $C_7H_5NaO_3$: sodium salicylate.

Table 2 – Publications that exploit flow analysis COD determination with electrochemical detection.

Detection device	Sample volume (μL)	Linear Range	Detection limit	Reference
TiO_2 photocatalytic sensor	3000	Up to 7.78 mg L^{-1}	1 mg L^{-1}	[23]
TiO_2 photocatalytic sensor	3000	0.12 – 8 mg L^{-1}	-	[24]
TiO_2 photocatalytic sensor	-	0.5 – 235 mg L^{-1}	-	[26]
F-PbO_2 electrode; Thin-layer amperometric detector	20	100 – 1200 mg L^{-1}	15 mg L^{-1}	[27]
F-PbO_2 electrode; Thin-layer amperometric detector	20	50 – 1200 mg L^{-1}	20 mg L^{-1}	[28]
Ti/TiO_2 electrode; Thin-layer photocatalytic amperometric detector	20	50 – 1000 mg L^{-1}	15 mg L^{-1}	[29]
Ti/TiO_2/PbO_2 electrode; Thin-layer photocatalytic amperometric detector	20	30 – 2500 mg L^{-1}	15 mg L^{-1}	[30]
Boron-doped diamond electrode	100	2 – 175 mg L^{-1}	1 mg L^{-1}	[32]

Table 3 – Publications that exploit flow analysis COD determination with chemiluminescence detection.

COD standard	Oxidant	Chemiluminescence reagent	Radicals generation	Metal ions interference removal	Linear Range	Detection limit	Reference
$C_6H_{12}O_6$	$KMnO_4$ in H_2SO_4	Luminol, H_2O_2	-	Cation ion-exchange column	4 – 4000 mg L^{-1}	2 mg L^{-1}	[33]
$C_6H_{12}O_6$	$KMnO_4$ in H_2SO_4	Luminol	-	EDTA	0.3 – 200 mg L^{-1}	0.3 mg L^{-1}	[34]
$C_6H_{12}O_6$	$K_2Cr_2O_7$ in H_2SO_4	Luminol, H_2O_2	-	EDTA	0.27 – 10 g L^{-1}	100 mg L^{-1}	[35]
KHP	-	Luminol	Ozonation with UV irradiation	Co	0.6 – 24 mg L^{-1}	-	[36]
$C_6H_{12}O_6$	-	Luminol	UV irradiation	Cation ion-exchange column or dilution of the sample	0.2 – 20 mg L^{-1}	0.08 mg L^{-1}	[37]
KHP	-	Luminol	UV irradiation, Quantum Dots	EDTA	1 – 35 mg L^{-1}	-	[38]

$C_6H_{12}O_6$: glucose; KHP: potassium hydrogen phtalate.

Quantum Dots Assisted Photocatalysis for the Chemiluminometric Determination of Chemical Oxygen Demand

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Abstract

A novel flow method for the determination of chemical oxygen demand (COD) is proposed in this work. It relies on the combination of a fully automated single interface flow system, an on-line UV photocatalytic unit and quantum dot (QD) nanotechnology. The developed approach takes advantage of CdTe nanocrystals capacity to generate strong oxidizing species upon irradiation with UV light, which fostered a fast catalytic degradation of the organic compounds. Luminol was used as a chemiluminescence (CL) probe for indirect COD assessment, since it is easily oxidized by the QD generated species yielding a strong CL emission that is quenched in the presence of the organic matter. The influence of the size and quantum yield of CdTe QD capped with 3-mercaptopropionic acid (MPA) prepared by a fast aqueous synthetic route was examined.

The proposed methodology allowed the determination of COD concentrations between 1 and 35 mg L^{-1}, with good precision (R.S.D. < 1.1%, n = 3) and a sampling frequency of about 33 h^{-1}. The procedure was applied to the determination of COD in certified reference materials and the obtained results showed an excellent agreement with the certified values, as well as good robustness and stability, allowing overcome some of the drawbacks associated with the standard dichromate method, such as long analysis time, expensive reagents, laborious operation and high solutions consumption.

Keywords: Single interface flow analysis; Chemical oxygen demand; Quantum dots; UV photocatalysis; Chemiluminescence.

1. Introduction

Chemical oxygen demand (COD), which represents the amount of oxygen consumed to completely chemically oxidize the organic water constituents to inorganic end products, is a key parameter for monitoring water quality upon assessment of the effect of polluting agents. This is an arbitrary empirical measurement typically carried out by subjecting the sample to oxidation either by potassium permanganate or potassium dichromate in acid solution under working conditions well-established by the official method [1]. Despite its widespread use, the official method for COD has several disadvantages such as: (i) the analysis is time-consuming (2–4 h being required for digestion plus additional time for the titration); (ii) handling is also considerable, increasing the probability of errors; (iii) a relatively high consumption of expensive (Ag_2SO_4) and toxic ($HgSO_4$, $K_2Cr_2O_7$) chemicals is verified [2].

To overcome these drawbacks several flow methodologies with different detection techniques (spectrophotometry [3-19], electrochemical [20-22], chemiluminescence [23-26]) were developed. Although they drastically reduce the analysis time, most of them still used the supra mentioned chemicals for promoting the oxidation of the organic matter. At the same time, different digestion strategies were proposed with these chemicals, including microwave [27-29] and UV irradiation [30-33].

In recent years, the photocatalytic decomposition of organic pollutants in water has received much attention. Recent investigations have proved that many semiconductors in aqueous solution will induce catalytic decomposition and often

61 complete mineralization of organic compounds [34-36]. A semiconductor particle is
62 believed to have a filled valence band separated from a vacant conduction band by
63 a gap whose energy is E_g. When irradiated with a light source, whose energy
64 exceeds E_g, an electron is promoted from the valence to the conduction band,
65 leaving behind a positive hole. The photocatalytic capacity depends on the
66 reactivity of the electron-hole pairs generated in the semiconductors. The electron
67 and the holes could recombine or could react with species in solution with
68 adequate redox potentials [37]. TiO_2 is one of the semiconductor materials most
69 used in photocatalysis either in suspension or as a coating in many supports like
70 quartz [38], stainless steel [39] or fiberglass [40].
71 Quantum dots (QD) are nanometer-scale semiconductor crystals and are defined
72 as particles with physical dimensions smaller than the exciton Bohr radius. The QD
73 core is made up of elements from the II–VI (e.g. CdSe, CdTe, CdS, ZnSe), III–V
74 (e.g. InP, InAs) or IV–VI (e.g. PbSe) group [41]. QD have been the subject of a
75 growing interest in recent years because of their superior photochemical
76 properties, such as higher quantum yields, photostability, tunability and broad
77 absorption and narrow emission, in comparison to conventional organic dyes [42].
78 Unlike TiO_2 quantum dot nanocrystals could be directly prepared in aqueous media
79 or could be made water-soluble by appropriate capping with hydrophilic ligands.
80 Several works have demonstrated that upon exposure to light QD have the
81 capacity to generate reactive oxygen species (ROS) in aqueous solution [43-44],
82 such as singlet oxygen, which supports their utilisation in photodynamic therapy
83 [45]. The bandgap of quantum dots could be also potentially high enough to

reduce oxygen to superoxide radical ($^{-\bullet}O_2$) or to oxidize water or hydroxide ions to hydroxyl radical ($^{\bullet}OH$) [43] (Figure 1). These radicals are the most reactive of the ROS and play an important role in oxidizing organic pollutants [37].

In this work CdTe quantum dots are used for the first time to promote the photocatalysis of several organic compounds upon irradiation with UV light, which is used as a means to assess sample COD. To carry this approach, and taking it account that it involves the measurement of short-lived species, a fully automated single interface flow system [46] comprising a photo-irradiation unit was developed. The solutions handling strategy associated with the concept of single interface flow analysis (SIFA) (47, 48), recently proposed by our group, relies on the establishment of an unique interface where all involved solutions are put in contact for reaction development, and does not require the utilisation of pre-set sample and reagent volumes which greatly simplifies system operation and control. The implemented analytical system included a chemiluminometric detector for monitoring the light emitted upon oxidation of luminol by the oxidizing species generated during CdTe quantum dots irradiation, which enables one to take advantage of the high sensitivity, wide dynamic working range, fast response and simple instrumentation provided by chemiluminescence measurements.

2. Experimental

2.1. Materials and Sample Preparation

All solutions were prepared with water from a Milli-Q system (specific conductivity < 0.1 $\mu S\ cm^{-1}$) and chemicals of analytical reagent grade quality. Reagents were not subject to any further purification.

A 1.0×10^{-2} mol L^{-1} luminol (Sigma-Aldrich) solution was prepared by dissolving 0.178 g in 100 mL of 0.1 mol L^{-1} sodium hydroxide.

A 5.0×10^{-2} mol L^{-1} ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich) solution was prepared by dissolving 9.306 g in 500 mL of water.

The 500 mg L^{-1} COD standard solution was prepared by dissolving 0.425 g of potassium hydrogen phthalate (KHP, Sigma-Aldrich) in 1 L of water. Working standard solutions were daily prepared by suitable dilutions with water.

For the synthesis of the quantum dots the subsequent reagents were used: sodium borohydride ($NaBH_4$, 99%), tellurium powder (200 mesh, 99.8%), cadmium chloride hemi(pentahydrate) ($CdCl_2 \cdot 2.5H_2O$, 99%), purchased from Sigma-Aldrich; 3-mercaptopropionic acid (MPA, 99%), from Fluka, and ethanol (99.5%), from Panreac.

Certified reference material (WasteWatR™ Demand standards) were purchased from ERA, Waters Company.

130 ## 2.2. Synthesis of CdTe Quantum Dots

131

132 MPA-capped CdTe QD were synthesized as described by Zou *et al* [49] with
133 some modifications. Briefly, NaHTe solution was prepared by reaction between
134 $NaBH_4$ and Te powder in N_2 saturated water. NaHTe was transferred into a second
135 flask containing 4.0 x 10^{-3} mol of $CdCl_2$ and 6.8 x 10^{-3} mol of MPA in a 100 mL N_2
136 saturated solution. The pH of the solution was adjusted to 11.5 by addition of 1 mol
137 L^{-1} NaOH. The molar ratio of Cd^{2+}:Te^{2-}:MPA was fixed at 1:0.1:1.7. The CdTe QD
138 size could be tuned by varying the heating time.
139 The purification of QD was performed by precipitation with ethanol. All the
140 fractions obtained were re-suspended in water maintaining the synthesis
141 concentration. Working solutions were daily prepared by suitable dilution with
142 water.

143

144 ## 2.3. Apparatus and Methods

145

146 The single interface flow system comprised four solenoid micro-pumps 120SP
147 (Bio-Chem Valve Inc., 10 μL per stroke), two 161T031 three-way solenoid valves
148 (NResearch) and a Camspec CL-2 chemiluminescence detector (Camspec)
149 equipped with a three-port 60 μL inner volume quartz flow cell. Reaction coil was
150 made of PTFE tubing (0.8 mm i.d.) and was inserted in a photo-irradiation unit that
151 consisted of a 15-W quartz UV lamp (λ_{max} = 254 nm). End-fittings, confluences and
152 connectors were also used.

153 A computer equipped with a PC-LABCard model PCL-711B (Advantech)
154 interface card was used for system control and for data acquisition and processing
155 by means of specifically developed software. A CoolDrive (NResearch) power drive
156 was used to operate both the solenoid micro-pumps and solenoid valves.
157
158 **2.4. Single Interface Flow Manifold and Procedure**
159
160 The developed flow manifold, pictured in Figure 2, comprised four solenoid
161 micro-pumps (P_1, P_2, P_3 and P_4) for inserting and propelling the sample and
162 reagent solutions. The repetitive micro-pump switching on/off created a pulsed
163 flowing stream in which the pulse volume corresponded to the micro-pump stroke
164 volume. Flow rate was determined by the micro-pumps frequency of actuation and
165 stroke volume. Two three-way (normally off) solenoid valves (V_1 and V_2) were used
166 to direct the flowing streams, either towards the analytical path of to waste.
167 The analytical cycle was started by establishing a baseline, which was
168 accomplished with the luminol and EDTA solutions. With this purpose P_1 and P_2
169 were simultaneously actuated for propelling these solutions through V_1 (off), the
170 detector and reaction coil towards V_2 (on) and waste (W). Subsequently, P_1 and P_2
171 were switched off and P_3 and P_4 were operated to insert the QD and the COD
172 standard solution (or sample) into the analytical path. These solutions when flowing
173 throughout V_2 (off) established a single reaction interface with the luminol and
174 EDTA solutions. The reaction interface was carried out back through the reaction
175 coil placed in the photo-irradiation unit and directed towards the detector and after
176 that to waste via V_1 (on). The reaction product formed as a consequence of the

177 mutual solutions intermingle produced an analytical signal, which was recorded as
178 a peak when the reaction interface passed through the flow cell.
179
180 **2.5. Certified Reference Material (CRM)**
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182 The certified reference material WasteWatR^{TM} Demand was prepared in
183 accordance to the given instructions. Working samples with COD values within the
184 linear application range of the developed methodology were prepared by suitable
185 dilution with water. Samples were analysed in triplicate.
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3. Results and Discussion

3.1. Reaction mechanism

The COD determination is based on the monitoring of the light emitted upon oxidation of luminol by the strong oxidizing species generated during CdTe quantum dots irradiation.

It is well known that the interaction between light and a QD can promote the delocalization of an electron (e-) from the valence band (V_b) to the conduction band (C_b).This occurs when the energy of the absorbed photons is higher or equal to the QD band gap. The formed Bohr exciton, an electron-hole pair, has a redox potential that it is dependent from the V_b and C_b potentials, and mainly from the flat band potential. It has been demonstrated that the photoirradiation of a QD aqueous solution can lead to the formation of reactive oxygen species [43], and we postulate that the formation mechanism is the following:

$$CdTe + hv \rightarrow CdTe\,(e\text{-}Cb + h\text{+}Vb) \quad (1)$$

$$e\text{-}Cb + h\text{+}Vb \rightarrow hvPL \quad (2)$$

$$e^{-}_{Cb} + h^{+}_{Vb} \rightarrow \Delta^{\circ} \quad (3)$$

$$H2O \rightarrow H3O + OH\text{-} \quad (4)$$

$$OH^{-} + h^{+}_{Vb} \rightarrow \cdot OH \quad (5)$$

$$O_2 + e^{-}_{Cb} \rightarrow \cdot\cdot O_2 \quad (6)$$

In equation (1) QD are irradiated with an electromagnetic radiation of energy higher than the band gap, forming the exciton (e^-_{Cb} + h^+_{Vb}). This energy can be returned to the system by radiative (2) or non-radiative processes (3). In presence of oxidable species, such as H_2O or OH^- from water dissociation (4) or normally adsorbed on QD's surface, h^+_{Vb} can be scavenged forming hydroxyl radicals (5). e^- can also be scavenged by oxidizing species, like O_2 normally present in water, leading to the formation of superoxide radical (6).

In the absence of organic matter all generated radicals are available to oxidize luminol. In this case the obtained chemiluminometric analytical signal is the highest possible. As the concentration of organic matter increases the scavenging of free radicals also increases, which results in a pronounced decrease of the luminol oxidation rate and therefore of the chemiluminescence emission obtained. Consequently, the analytical signal will diminish with the increase of organic matter contents allowing the indirect assessment of COD.

The formation of reactive oxygen species is also facilitated by the presence of EDTA. This compound interacts with the QD surface, probably by chelating the surface cadmium atoms, and is easily oxidized by the photogenerated holes [50]. The absorption of EDTA has the main effect of alter the flat band potential and consequently the V_b and C_b potentials favouring the oxidation of some chemicals species [51, 52].

3.2. Quantum Dots (QD)

Six fractions of quantum dots were obtained in the synthesis process previously described. The size of the quantum dots in these fractions decreased from 3.1 nm in the first fraction to 2.3 nm in the last fraction.

The influence of quantum dots size was evaluated by using a 1.0×10^{-2} mol L^{-1} luminol solution, a 5.0×10^{-2} mol L^{-1} EDTA solution and 50 and 100 mg L^{-1} COD solutions. The reaction coil had 50 cm length and the flow rate was 1.5 mL min^{-1}.

The results obtained showed that the highest sensitivity was achieved for the QD of greater size. The addition or subtraction of just a few atoms to the quantum dot has the effect of altering the boundaries of the band gap. Since quantum dots with higher size have a smaller band gap, electrons required inferior energy to be raised to the conduction band which facilitates the formation of the positively charged holes in the valence band, which are subsequently responsible for the generation of the oxidizing species.

3.3. Optimization of the SIFA manifold

In the development of the single interface flow methodology several experiments were carried out in order to improve system performance, namely in terms of sensitivity, accuracy, precision and sampling rate. These features influenced the choices made during the optimisation of the systems, which was carried out using the univariate method. Since no well-defined sample or reagent volumes were

271 used, these parameters were not subject to evaluation, thus simplifying the
272 optimisation process.
273 The physical parameters were evaluated using the quantum dots of higher size,
274 a 1.0×10^{-2} mol L^{-1} luminol solution, a 5.0×10^{-2} mol L^{-1} EDTA solution and COD
275 solutions with concentrations between 0 and 50 mg L^{-1}.
276 Influence of flow rate used to propel the solutions was assessed between 1.5 and
277 3.0 mL min^{-1}. Increasing flow rate increased the magnitude of the analytical signal
278 up to 2.0 mL min^{-1}. Higher flow rates lack repeatability. A 2.0 mL min^{-1} flow rate
279 was then selected.
280 The length of the serpentine reaction coil was also assessed. The evaluation of
281 the analytical signals obtained with reaction coils ranging from 25 to 100 cm
282 revealed that the analytical signals were higher for a coil length of 50 cm.
283 Consequently, a 50 cm reaction coil was selected for further experiments.
284 EDTA was used to avoid the effects caused by metal ions [25, 26]. Influence of
285 EDTA concentration was investigated for concentrations ranging from 1.0×10^{-2} to
286 1.0×10^{-1} mol L^{-1}. The obtained analytical signals showed a pronounced increase
287 up to EDTA concentration values of 5.0×10^{-2} mol L^{-1}. Concentration EDTA values
288 of 1.0×10^{-1} mol L^{-1} and higher led to the precipitation of the quantum dots.
289 Therefore, a 5.0×10^{-2} mol L^{-1} EDTA solution was used in the subsequent
290 experiments.
291 Luminol concentration was also assessed for concentrations between 5.0×10^{-3}
292 and 5.0×10^{-2}. The evaluation of the analytical signals obtained revealed that the

293 analytical signals were higher for a concentration of 1.0×10^{-2} mol L^{-1}. Accordingly,
294 a 1.0×10^{-2} mol L^{-1} concentration was then selected.
295
296 **3.4. Analytical features of merit**
297
298 Under the analytical conditions exhibited in Table 1, a linear working
299 concentration range between 1 and 35 mg L^{-1} was obtained. The analytical curve
300 was typically expressed as:
301
302 $h = -0.1338\ C + 10.022$
303
304 Where h = peak height (cm) and C = COD concentration (expressed in mg L^{-1}).
305 The correlation coefficient was -0.9993.
306 Furthermore, the repeatability was good, with relative standard deviations
307 between 0.2 and 1.1% (*n*=3). The proposed SIFA system allowed about 123
308 determinations per hour, corresponding to the analysis of about 33 samples per
309 hour (considering the time required for sample replacement), which represents a
310 significant improvement regarding the official method that takes at least 2 hours for
311 the determination of the COD of just one sample.
312 In order to evaluate the applicability and the accuracy of the proposed
313 method, certified reference material was analysed. The results (Table 2) showed
314 an excellent agreement between the certified value and the experimental value
315 obtained with the SIFA method.

316

317 ## 4. Conclusions

318

319 This work proposed a novel flow analysis approach for COD determination,
320 which exhibit noteworthy advantages regarding the available methodologies.
321 Effectively, coupling for the first time the concept of photocatalysis to a fully
322 automated single interface flow system by means of the incorporation of an on-line
323 UV irradiation unit demonstrated the feasibility and robustness of this flow-based
324 methodology and confirms its versatility and ease of operation and control. In
325 addition, not only it made it possible to eliminate the long reflux time normally
326 required allowing a good sampling rate (33 h^{-1}), but it was also able to prevent the
327 use of tedious manual procedures that are more prone to the occurrence of errors.
328 Moreover, the implemented reaction scheme avoided the use of expensive
329 reagents (Ag_2SO_4) and exploited, for the first time, the analytical potential and
330 reactivity of CdTe quantum dots (in aqueous medium) for COD determination,
331 showing that upon irradiation with UV light they provide a fast photodegradation of
332 the organic compounds, making this work an attractive and promising start point for
333 future trends and developments.

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463 **Figures captions:**

464

465 **Figure 1** – Schematic representation of the production of hydroxyl radicals in a QD
466 nanocrystal.

467

468 **Figure 2** – Single Interface Flow Manifold for the determination of COD. Legend:
469 P_1, P_2, P_3, P_4: solenoid micro-pumps (10 μL per stroke); X, Y: confluences; V_1, V_2:
470 solenoid valves; PIU: photo-irradiation unit (15-W quartz UV lamp, λ_{max} = 254 nm),
471 RC: 50-cm reaction coil; S: COD standard or sample; D: chemiluminescence
472 detector; W: waste.

473

Table 1 – Range of values used in dimensioning the SIFA system, and selected operating conditions for the COD determination.

Parameter	Range	Selected value
Flow rate (mL min^{-1})	1.5 – 3.0	2.0
Reaction coil length (cm)	25 – 100	50
EDTA concentration (mol L^{-1})	1.0×10^{-2} – 1.0×10^{-1}	5.0×10^{-2}
Luminol concentration (mol L^{-1})	5.0×10^{-3} – 5.0×10^{-2}	1.0×10^{-2}

Table 2 – Results obtained in the analysis of COD of commercially available certified reference material.

WasteWatR™ Demand	Certified value (mg L^{-1})	QC PALS [a] (mg L^{-1})	COD determined [b] (mg L^{-1})
Sample 1	146	123 – 162	146.2 ± 1.0
Sample 2	113	96.3 – 127	113.7 ± 0.3

[a] Quality Control Performance Acceptance Limits (95% confidence interval);

[b] Values expressed as mean ± standard deviation.

Figure-1
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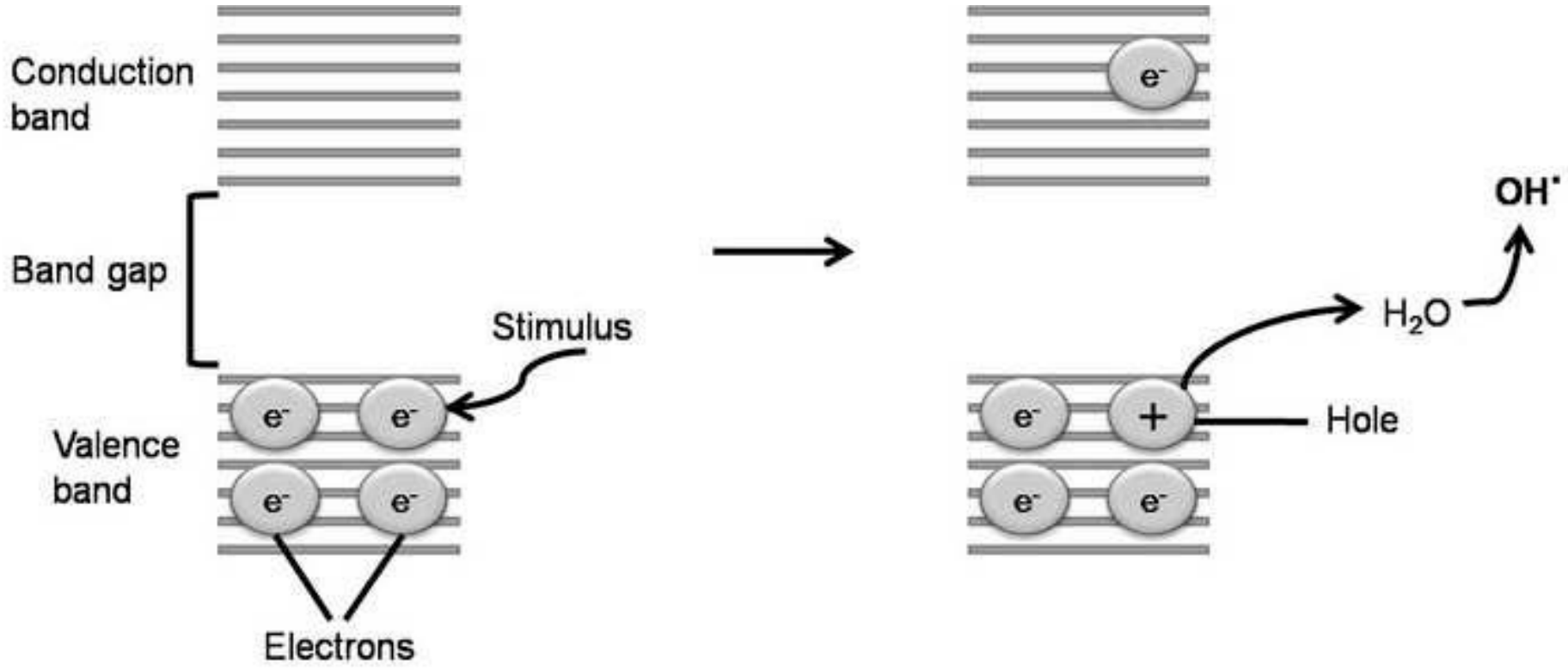

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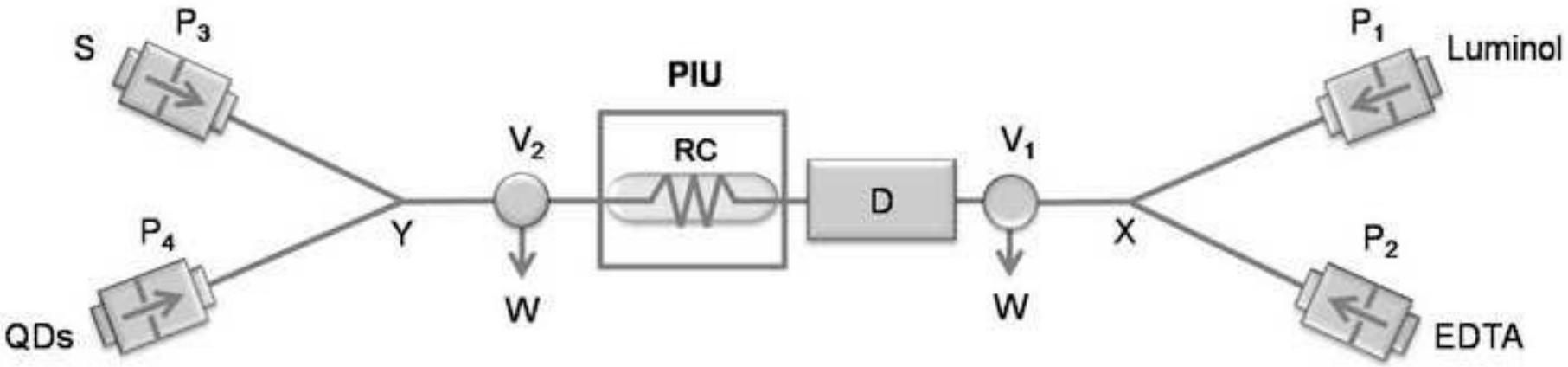

CAPÍTULO 8

Conclusões Gerais

Os sistemas SIFA representam uma nova estratégia de gestão de fluidos, a qual diverge significativamente do conceito de análise em fluxo, na medida em que não implica a inserção de volumes definidos de amostra e reagentes nas montagens analíticas, mas sim o estabelecimento de uma interface única de reacção entre a amostra e os reagentes. Desta forma, a optimização das montagens apresentadas foi relativamente simples, em virtude da minimização da influência dos parâmetros volume de amostra e de reagentes.

Para demonstrar a versatilidade e o potencial analítico dos sistemas SIFA, nas montagens analíticas foram incluídos diferentes tipos de dispositivos de inserção e propulsão de soluções (micro-bombas solenóides e buretas automáticas), assim como, diferentes tipos de detectores (espectrofotométrico, quimioluminométrico, fluorimétrico), localizados quer numa posição central, quer numa posição terminal da montagem analítica.

Utilizando os sistemas SIFA propostos foi possível explorar, pela primeira vez, novas reacções para a determinação de analitos como manitol (baseada na sua actividade "*scavenger*" de radicais hidroxilo), hormonas da tiróide e lansoprazol (utilização de aceitadores-π).

Para além disso, foi também exequível a implementação, num sistema SIFA, do método de Job ou método das variações contínuas que permitiu estabelecer a estequiometria dos complexos formados, assim como, do conceito de avaliação da exactidão, baseado na média dos resultados obtidos através de dois métodos *quasi*-independentes originando um resultado mais preciso e exacto e, ao mesmo tempo, expandindo o conceito de SIFA ao estabelecer duas interfaces de reacção.

Foi também implementado, pela primeira vez em análise em fluxo, uma extracção aquosa bifásica para a pré-concentração de chumbo. O facto desta operação unitária ser realizada num sistema de fluxo já resolve várias limitações relacionadas com o tempo de análise e com a manipulação por parte do operador. Convém ainda enfatizar a importância deste tipo de extracção em termos ambientais, uma vez que se baseia na utilização de duas fases aquosas imiscíveis, não fazendo uso dos solventes orgânicos tão comuns nas extracções líquido-líquido convencionais. Adicionalmente, verifica-se que a interface única estabelecida é utilizada quer como interface de extracção, quer como interface de reacção.

Num último trabalho desenvolvido no âmbito desta dissertação, alia-se, pela primeira vez, a foto-catálise com a nanotecnologia *quantum dot* para a determinação quimioluminométrica da carência química de oxigénio, o que é algo realmente inédito.

Explorando, desta forma, a capacidade dos *quantum dots* de CdTe para gerar espécies oxidantes, quando irradiados com luz UV, realizava-se a foto-catálise da matéria orgânica presente nas amostras. Esta abordagem permite obviar vários problemas associados com a metodologia oficial para a determinação de CQO, nomeadamente, longo tempo de análise, operações fastidiosas e consumo de reagentes dispendiosos e tóxicos.

Assim, através dos trabalhos desenvolvidos procurou-se estudar e caracterizar o desempenho analítico dos sistemas SIFA em diferentes situações analíticas, introduzindo em todos eles conceitos novos que contribuem de alguma forma para o desenvolvimento científico.